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EXAMINING THE NATURE OF ADHESION BETWEEN CULTURED THYMIC STROMAL CELLS AND $CD45^{+/+}$ OR $CD45^{-/-}$ CELL LINES

by

Kara Lea Allanach

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

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Date: 8 Aug 2003

ABSTRACT

Thymic selection involves interactions between developing thymocytes and thymic stromal elements. The transmembrane protein tyrosine phosphatase CD45 may play a role, as $CD45^{-/-}$ mice display a major block in thymocyte development (15, 59, 67). Also, the size and carbohydrate content of the extracellular domain of CD45 is regulated during thymic development (51, 111). CD45 may play a role in the regulation of thymic development through direct interactions or a more indirectly through regulation of other surface molecules such as integrins. We observe CD45-dependent adhesion between cultured thymic stromal cells and T-lymphoma cells, which can be inhibited by EDTA and by specific carbohydrates, indicative of a cation-dependent carbohydrate-based interaction. However, this adhesion can also be inhibited by a monoclonal antibody against the integrin VLA-4, supporting indirect involvement through integrin regulation. It is entirely possible that CD45 may be involved in both types of adhesion. Further studies will be required to clarify this issue.

For Austin

You are my inspiration

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Getting to this point in my life has been an incredible journey. Seven years ago when I started university, I didn't even know what a 'significant digit' was. Now I have written and successfully defended an M.Sc. thesis. Sometimes when I look back on these years I can't imagine how I ever made it through. One thing I know for sure is that without the support of my family and friends I certainly would not have been able to do it.

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LIST OF ABBREVIATIONS

2D: two-dimensional

3D: three-dimensional

AP-1: activator protein-1

APC: antigen presenting cell

β_2m: β-2 microglobulin

BW5147: T-lymphoma cell line

C2GnT: core 2 β-1,6-*N*-acetylglucosaminyltransferase

CD: cluster designation

c-kit: CD117; stem cell factor receptor

CRD: carbohydrate recognition domain

Csk: C-terminal Src-family kinase

CTL: cytotoxic T lymphocyte

D1: membrane proximal CD45 phosphatase domain

D2: membrane distal CD45 phosphatase domain

DC: dendritic cell

DCS: defined calf serum

DC-SIGN: dendritic cell specific ICAM-grabbing non-integrin

DMEM: Dulbecco's modified eagle medium

DN: double negative

DP: double positive

ECM: extracellular matrix

EDTA: Ethylenedinitrilo-tetraacetic acid

EGFR: epidermal growth factor receptor

FACS: fluorescence activated cell sorting

FAK: focal adhesion kinase

FCS: fetal calf serum

FITC: fluorescein isothiocyanate

FN: fibronectin

FRET: fluorescence resonance energy transfer

FTOC: fetal thymic organ culture

Fyn: Src-family kinase

GII: glucosidase II

Gln: glutamine

Hck: Src-family kinase

ICAM: intracellular adhesion molecule

IgG: immunoglobulin G

IL-2Rα: interleukin-2 receptor α-chain

ITAM: intracellular tyrosine-based activation motif

kDa: kilodalton

LAT: linker for activation of T cells

LFA-1: leukocyte functional antigen-1

mAb: monoclonal antibody

MFI: mean fluorescence intensity

NEAA: non-essential amino acids

PBS: phosphate buffered saline

Pen/Strep: penicillin / streptomycin

Pgp-1: phagocytic glycoprotein-1

PLCγ-1: Phospholipase C- γ-1

PMA: Phorbol 12-myristate 13-acetate (a.k.a. TPA)

PNA: peanut agglutinin

PNGaseF: peptide: N-glycosidaseF

pre-T-α (pTα): pre-T-α-chain

PTP1B: protein tyrosine phosphatase 1B

PTPase: protein tyrosine phosphatase

RAG: recombinase activating gene

RT-PCR: reverse transcriptase-polymerase chain reaction

SAKR: T-lymphoma cell line

SCID: severe combined immunodeficiency

SH2: Src-homology 2

TCR: T-cell receptor

TEC: thymic epithelial cell

tgε26: CD3ε-expressing transgenic mouse line

T_H: T-helper cell

TN: triple negative

TSC: thymic stromal cell

UCHL-1:anti-CD45R0 antibody

UL: upper left quadrant

UR: upper right quadrant

VCAM: vascular cell adhesion molecule

VLA: very late antigen

Y_{394}: tyrosine 394 of Lck

Y_{505}: tyrosine 505 of Lck

Y505F: Lck with phenylalanine substituted for tyrosine 505

Zap-70: zeta-chain associated protein of 70 kDa

CHAPTER I

INTRODUCTION

THE IMMUNE SYSTEM

Protection against the invasion of foreign pathogens is provided by the immune system. The immune system is remarkable in its complexity and its efficiency. It must be able to recognize and eliminate foreign invaders while maintaining tolerance to self proteins and cells in order to avoid autoimmunity. This requires a high degree of specificity, as differences between self and foreign antigens can be extremely subtle. Along with this specificity, however, extensive diversity is also necessary, as the number of potential insults the body is exposed to within a lifetime can be staggering.

Innate and Adaptive Branches of the Immune System

The immune system is composed of two major branches: the innate or non-specific branch and the adaptive or specific branch. The innate immune system represents the body's first line of defense against foreign invaders. It includes the skin and mucous membranes, which provide mechanical barriers to pathogen entry. It also includes physiological barriers, such as body temperature, low pH in the stomach, and anti-pathogenic enzymes and proteins such as lysozyme, interferon and complement. Central to the innate immune response is inflammation. This response is initiated when tissues are wounded

or foreign pathogens are successful in breaking through the initial mechanical and physiological barriers. When this occurs, the body responds by increasing vascular permeabilization in the vicinity of the injury. This allows the release of antimicrobial proteins, as well as the mobilization of phagocytic monocytes, neutrophils, macrophages and dendritic cells from the bloodstream directly to the site of injury. These phagocytic cells are able to directly endocytose, kill and digest foreign pathogens. Uptake of foreign organisms by these cells also provides a crucial link between the innate and adaptive branches of immunity.

Professional antigen presenting cells (APCs) such as dendritic cells, following encounter with a foreign antigen, undergo molecular changes to allow them to digest foreign antigens for presentation on their surface in the context of Major Histocompatibility Complex Class II (MHCII) proteins, travel to the spleen and lymph nodes and activate T-helper (T_H) cells specific for the particular antigen/MHCII complex that they carry. These T_H cells can then go on to activate B cells to produce antibodies or cytotoxic T lymphocytes (CTL) to directly lyse infected self cells.

The diversity and specificity of the adaptive immune system is possible because each individual B and T cell expresses a unique antigen-recognition receptor on its surface. Thus the millions of lymphocytes in the body, as a population, carry millions of unique antigen specificities. When one of these lymphocytes encounters its particular antigen it undergoes a vigorous round of proliferation to produce a large number of "clones" with the same specificity as

the original lymphocyte. These clones act as specific effectors to rid the body of the antigen that initiated the clonal expansion.

The ability of the immune system to recognize such a large array of antigens is beneficial, provided that the specific proliferation-inducing antigen is indeed a foreign one. A clonal effector response against the body's own elements could have disastrous results, resulting in autoimmunity. In order to avoid an autoimmune response, T cells undergo an elaborate developmental process within the thymus before they are released into peripheral circulation. The T cells that are found in the peripheral blood system have the diversity to recognize a vast array of foreign antigens, but also the specificity to be able to differentiate between foreign and self. These characteristics are developed through a rigorous selection process that occurs most efficiently within the thymus (71). During thymic development, T cells that do not recognize self MHC with high enough affinity die by neglect, those that react with self-MHC with very high affinity are induced to die by apoptosis, and only those capable of recognizing self-MHC at an appropriate affinity are allowed to undergo the later selection events culminating in the entry into the periphery.

THYMOCYTE DEVELOPMENT

T-cell development occurs along a well-defined pathway, with the stages being defined by the expression of various surface molecules (figure 1). A number of studies have been undertaken to outline the importance of various

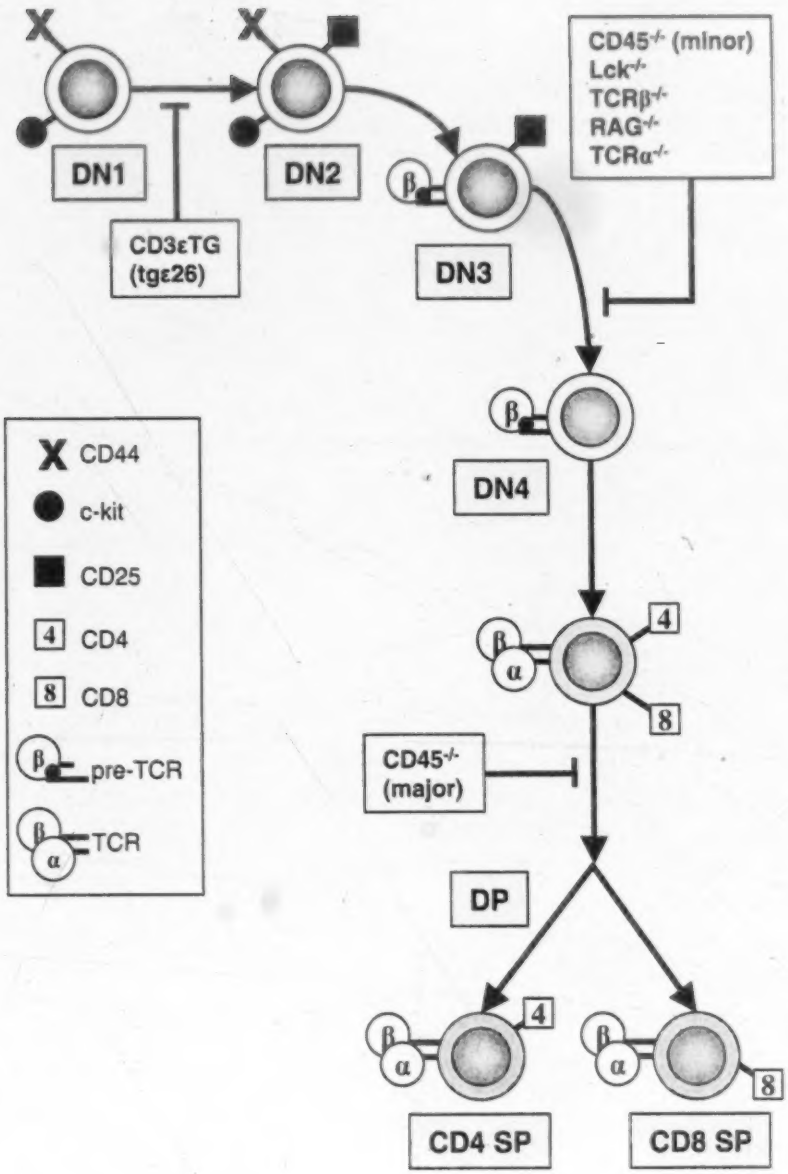


Figure 1: The Stages of Thymocyte Development.

molecules in the process of thymocyte development, and in the broadest sense, developmental stages can be differentiated based on the expression of the co-receptors CD4 and CD8. Some of the molecules believed to play a central role in thymocyte development are CD45, Lck and the TCRβ chain, along with other molecules known to be involved in membrane-proximal TCR signaling.

The Double Negative Stage of Thymocyte Development

When immature thymocytes first enter the thymus from the bone marrow they do not express CD4 or CD8, and are therefore known as double negative (DN) cells. Some groups refer to this thymocyte subset as triple negative (TN), because the cells also do not express the CD3/TCR complex (46). As these cells move through the thymus they interact with thymic stromal cells, resulting in the upregulation of the expression of both CD4 and CD8. Thymocytes at this stage are called double positive (DP) cells. These double positive cells have undergone productive rearrangement of the TCRβ chain and start to express a pre-TCR complex composed of TCRβ, a pre-Tα chain and the CD3 complex (34). Through a number of further developmental interactions, DP cells lose expression of either CD4 or CD8 to become single positive (SP) thymocytes that are ready to leave the thymus to enter the periphery as naïve T cells. It is important to note at this point that most of the thymocytes that achieve a DP status do not end up completing the developmental process, and are actually induced to die within the thymus. During the DP stage thymocytes are subjected to processes known as positive and negative selection, and only those

thymocytes with the ability to recognize self-MHC complexes while still maintaining tolerance to self are allowed to complete the developmental process and progress to the SP stage.

Flow cytometric analysis of surface CD4/CD8 expression on *ex vivo* thymocytes reveals a specific distribution pattern of thymocytes expressing these co-receptors. In a normal thymus approximately 5% of thymocytes are DN, 80% are DP, 10% are CD4 SP and 5% are CD8 SP cells (17). Further characterization of surface markers has revealed a more detailed stepwise maturational sequence within the DN stage. Expression of the surface molecules CD44 (Pgp-1) (63), CD25 (IL-2Rα) (18) and c-kit (CD117, stem cell factor receptor) (46) were analyzed by flow cytometry. These studies determined that thymocytes of the DN stage can be further divided into 4 subsets termed DN1-DN4. These subsets are defined by the stepwise expression of these surface markers, according to the following maturational sequence: DN1, $CD44^+/CD25^-/c\text{-}kit^+$ → DN2, $CD44^+/CD25^+/c\text{-}kit^+$ → DN3, $CD44^-/CD25^+/c\text{-}kit^-$ → DN4, $CD44^-/CD25^-/c\text{-}kit^-$(figure 1) (45).

Rearrangement of TCRβ genes occurs within the DN3 subset, when the thymocytes downregulate the expression of both CD44 and c-kit (44). TCR genes are rearranged, or assembled by V(D)J recombination, a process that is central to the development of the extensive diversity of the T cell population (25). Recombinase activating gene-1 (RAG-1) (74) and RAG-2 (98) control this process. If the TCRβ chain cannot be rearranged, as is the case in RAG^{NULL} (74, 98) and TCRβ (73) mutant mice, thymocytes with a DN3 phenotype accumulate,

and thymocytes of later developmental stages are absent or their numbers are significantly decreased. This DN3 block can be rescued by the expression of a functionally rearranged TCRβ chain (29), implicating its rearrangement as an important checkpoint during the development of thymocytes. In particular, it has been shown that a functional TCRβ chain rearrangement is required for the progression from the DN→DP stage.

Thymocyte Development and Signaling through the pre-TCR

In a process known as β-selection, only those cells with functionally rearranged TCRβ chains are allowed to differentiate from the DN→DP stage (29). Once a cell rearranges a functional TCRβ chain this TCRβ associates with a nonpolymorphic form of the TCRα chain, the pre-T-α (pTα), and the CD3 proteins to form a pre-TCR complex (49). Thymocytes that are not successful in their TCRβ rearrangements are induced to die by apoptosis, probably due to lack of survival signals transmitted through the pre-TCR (33). Although the direct mechanisms are not clearly understood, signals transmitted through the pre-TCR complex are responsible for the initiation of numerous events leading to further thymocyte maturation, including rescue from apoptosis, proliferation, downregulation of CD25 expression, upregulation of both CD4 and CD8 expression, cessation of TCRβ gene rearrangement (allelic exclusion), and initiation of mature TCRα rearrangement (70). Analyses of mouse models with deletions or mutations in a number of molecules involved in membrane-proximal signaling, including Lck, Fyn (113), ZAP-70, Syk (21, 104), SLP-76 (23, 83) and

LAT (122) have shown that signals are transmitted through the pre-TCR in a similar manner to those transmitted through the mature TCR. The observed thymic developmental phenotypes observed in these mouse models outline the importance of a link between the pre-TCR complex with the classical membrane-proximal TCR signaling machinery in the progression from the DN→DP stages.

In support of this requirement for normal membrane-proximal TCR signaling, CD45 gene-ablated mice exhibit a defect in thymocyte development. These mice display a block at this DN→DP stage as well, although not a complete block. The major block observed in $CD45^{-/-}$ mice actually occurs at the DP→SP transition (15, 59, 67). This is somewhat surprising, as the most well-known function of CD45 is to activate Lck, the kinase responsible for the initiation of the membrane-proximal TCR signaling cascade. $Lck^{-/-}$ mice display a complete block in thymocyte development at the DN→DP (DN3) stage. If the sole function of CD45 in TCR signaling is to activate Lck, one would expect to see the same phenotype in a $CD45^{-/-}$ mouse as that seen in the $Lck^{-/-}$. The fact that thymocytes from the $CD45^{-/-}$ mice are capable of development beyond the DN3 stage suggests that CD45 may play another role during thymocyte development, in addition to the activation of Lck.

Positive and Negative Selection: DP→SP transition

Thymocyte development culminates in the final, crucial transition from the DP→SP stage. This step is particularly critical, as it is during this transition that the specificity and the functional phenotype of developing thymocytes is finally

decided. Ultimately, the subset of thymocytes that are best suited to function within the host environment will be allowed to mature and become peripheral T lymphocytes. The events responsible for this final decisive step are known as positive and negative selection, both of which are strongly dependent upon interactions between the TCR and MHC/peptide complexes. It is clear that MHC-presenting thymic stromal cells, the non-lymphocytic component of the cells within the thymus, are required for this final developmental stage. Through adoptive transfer experiments it was shown that T cells preferentially recognize MHC of the haplotype encountered during the developmental process, and not necessarily their own genetic haplotype (12, 13, 36, 101, 123, 124).

There is an apparent paradox in this DP→SP transitional stage of thymic development: both positive selection (survival) and negative selection (apoptosis) are mediated by TCR/MHC interactions. How can it be that these opposing signals can both be mediated by the same interaction? Although many of the specific interactions involved have not yet been clearly elucidated, most of the available evidence supports the theory that quantitative signaling, or the avidity of the TCR for self-MHC expressed on thymic stromal cells determines whether a thymocyte is positively or negatively selected. Essentially, in this model all signals received through the various TCR complexes on the surface of the thymocyte are integrated intracellularly, and if the collective signals received reach a certain threshold the thymocyte is positively selected, a survival signal is transmitted, and the thymocyte is allowed to continue to differentiate.

As with the other stages of thymocyte development, positive selection is defined by the expression of specific cell surface markers. Early DP thymocytes, prior to the onset of positive selection, are $TCR^{lo}/CD69^{-}$. Following TCR/MHC-peptide interaction facilitating positive selection, thymocytes become $TCR^{hi}/CD69^{+}$. Thus CD69 is used as a marker to identify cells that have undergone positive selection (58). If the level of signaling achieved does not reach the positive selection threshold, the thymocyte does not receive a survival signal, and is destined to die by neglect because of the lack of survival signal transmission. According to this model, positive selection could just as efficiently be achieved in response to numerous weak signals as it could in response to fewer strong signals. Negative selection is achieved through a similar process, although the signaling threshold required for the initiation of negative selection is considerably higher. This model allows for the possibility that other molecules, such as co-receptors and/or adhesion molecules could play a role in thymocyte selection, perhaps by increasing the half-life of the TCR-peptide/MHC interaction. Thus, the TCR-peptide/MHC interaction may not be entirely responsible for the selection of the mature T cell repertoire (91).

Evidence for the involvement of other molecules in the thymic selection process come from studies using transgenic mice expressing the 2C TCR, which is specific for peptide plus MHCI of the L^d haplotype. Thymocytes bearing the 2C TCR are normally positively selected in the presence of the $H\text{-}2K^b$ MHC, through a low-affinity interaction (94). If expression of the MHCI-specific co-receptor CD8 is increased on thymocytes from these same mice, however, the thymocytes

undergo negative selection in response to interaction with K^b, supporting the idea that an increase in the avidity of interaction between thymocytes and selecting cells can lead to negative selection (62, 85). Further studies show that thymocytes that undergo selection in the absence of co-receptor activity are induced to become autoreactive if the co-receptors are subsequently allowed to act (97).

Studies utilizing TCR transgenic mice on an MHC-deficient background have provided direct evidence to support the avidity model of thymocyte selection. Two separate groups initially showed that thymocytes from TCR transgenic, MHC-deficient mice, in the presence of specific peptide ligand undergo positive selection in response to low peptide concentrations, and negative selection in response to high concentrations of the same peptide (9, 92). These findings agree very well with the avidity theory of thymocyte selection. Further support for this model was provided using the OVA peptide-specific TCR transgenic mouse model. Direct measurement of the affinity of the TCR and various peptide ligands was performed. These measurements showed that indeed the ligands involved in the positive selection of thymocytes exhibited lower affinity for the TCR than did the negatively selecting ligands (2).

There was some early controversy surrounding which specific types of thymic stromal cells are required for MHC/peptide presentation to developing thymocytes (4, 53, 100). The first experiments using MHC-mismatched bone marrow chimeras suggested that the radio-resistant thymic stroma is responsible for the presentation of signals required for thymocyte maturation. In these

studies irradiated mice of one MHC haplotype (recipient mice) were reconstituted with bone marrow from mice of a different MHC haplotype (donor mice). Cellular proliferation is blocked in the irradiated mice, so thymocytes from these mice cannot undergo normal development. The thymic epithelia, however, is relatively resistant to irradiation as it is composed of differentiated cells. In this system, after the transferred thymocytes were allowed to develop, mature T cells with TCR specific for the MHC haplotype of the recipient mice were found. For a time it was accepted that MHC presented on thymic epithelia was required to mediate positive selection of thymocytes.

However, subsequent studies in $\beta_2$microglobulin-deficient (and therefore negative for surface MHCI expression) recipients reconstituted with fetal liver cells (which are positive for surface MHCI expression) revealed that $CD8^+$ T lymphocytes could develop, although not to the same degree of efficiency as observed in wild type mice. This suggests that hematopoietic cells can mediate positive selection of MHCI-restricted cells (14). This result is difficult to interpret, however. First, as a small number of $CD8^+$ T cells are able to develop in β_2m-deficient mice (6), it is possible that the presence of the exogenous hematopoietic cells simply facilitate the expansion of this small population. Another potential explanation is that thymic epithelial cells are able to mediate thymocyte development because they present MHC along with essential, but currently undefined costimulatory molecules. These unknown costimulatory molecules would still be present on β_2m-deficient thymic epithelia. So the presentation of MHC on the surface of exogenous hematopoietic cells, along with

the other molecules on the thymic epithelia may be responsible for thymic selection in this model.

Thymic Crosstalk

Not only do the thymocytes depend upon thymic stromal elements for their proper differentiation, thymic stromal cells also depend upon the developing thymocytes for their proper organization. These 'lymphostromal' interactions are required for the development of the unique thymic microarchitecture. This process is known as thymic crosstalk (3, 112).

Anatomically, the thymus is organized into 2 main regions: the cortex, which is the outer region of the thymus, and the inner region, known as the medulla. The major stages of thymocyte differentiation and education occur in the cortical region, and the final maturation stages prior to the exit to the periphery occur in the medulla. As they move through these microenvironments, thymocytes follow a specific migrational pathway. Bone marrow-derived T cell progenitors enter the thymus at the cortico-medullary junction and then migrate through distinct microenvironments within the cortex, moving from the subcapsular cortex to the deep cortex, then to the cortico-medullary junction and into the medulla.

The thymic microenvironments are composed of a network of heterogeneous, MHCII-expressing cell types, including cortical and medullary epithelial cells, fibroblasts, macrophages and dendritic cells. In a normal thymus, these cells form a three-dimensional (3D) mesh-like arrangement, allowing

thymocyte migration throughout the structure, and facilitating close contact with the various types of stromal cells. This cellular arrangement is unique, particularly for the epithelial cells. In most other organs, epithelial cells are arranged in a two-dimensional (2D) manner, anchored to a basement membrane to form epithelial sheets, creating barriers in and between organs.

Studies using immunodeficient transgenic mice have provided some information regarding the inter-relatedness of thymocyte development and the formation of the 3D stromal arrangement (112). CD3ε transgenic mice (tgε26 mice) express a very high copy number of the human CD3ε gene, which presumably leads to premature TCR signaling and thus interference with thymocyte development (112). Analysis of thymocytes from these mice reveals a developmental block in the DN1 stage ($CD44^+/CD25^-/c\text{-}kit^+$). The thymi of these mice are poorly organized, lacking a distinct border between the cortical and medullary regions. Perhaps more importantly, thymic epithelial cells within the thymi of these mice do not assume the normal 3D arrangement, and are largely arranged in 2D sheets anchored to a basement membrane. Another strain of immunodeficient mice, the RAG^{Null} mice, display block in thymocyte development at the DN3 stage ($CD44^-/CD25^+/c\text{-}kit^-$) (74). This is a slightly later stage than the one seen in the tgε26 mice. Thymi from these mice have normally developed cortical regions with epithelial cells arranged in a 3D manner, but medullae are still absent. When RAG^{Null} bone marrow was transplanted into the tgε26 mice, thymic development in the chimeric mice progressed to the same point as was seen in the RAG^{Null} mice (112). Thymic organization was also identical to that

seen in the RAG^{Null} mice, indicating that the signal for thymic epithelial cells to assume a 3D arrangement is presented somewhere between the DN1 and DN3 developmental stages. These RAG^{Null}→tgε26 chimeras were subsequently transplanted with wild-type bone marrow cells, resulting in fully normal thymocyte development and also in the correct organization of the thymic medullary compartment. These experiments together indicate that the presence of developing thymocytes directs the formation of the thymic microenvironments in a stepwise fashion, with the development of the cortical compartment preceding the development of the medullary region (112).

T-CELL SIGNALING

Peripheral T cells are the result of the extensive process of thymic selection. The end result of this selection is T cells expressing a TCR capable of recognizing foreign antigen presented as a peptide in the groove of either MHCI or MHCII, while still maintaining tolerance to self-peptides that may be presented on the same MHC. Recognition of foreign antigen results in activation of the T cell through an extensively studied and well-characterized pathway (121) (figure 2). In general, following interaction with a relevant peptide/MHC complex the immunoreceptor tyrosine-based activation motifs (ITAMs) in the TCR complex become phosphorylated on tyrosine residues by the Src-family kinase Lck ($p56^{Lck}$). This phosphorylation provides a docking site for SH2-domain-containing proteins such as Zap-70 (19). The newly recruited kinase, Zap-70, is then phosphorylated by Lck, and proceeds to phosphorylate various downstream

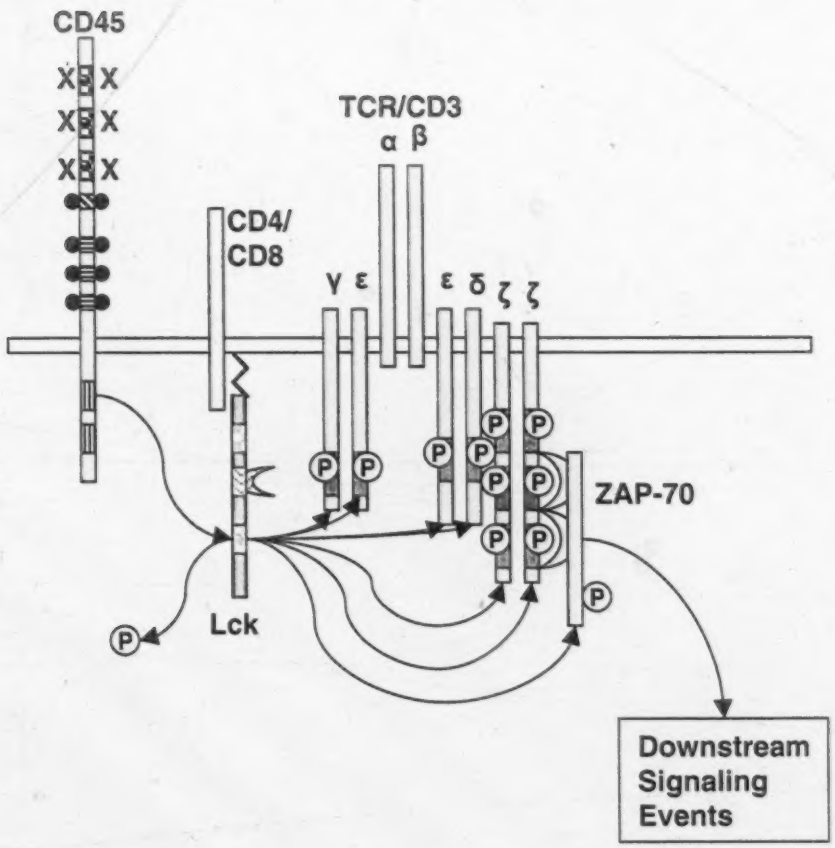


Figure 2: Membrane-Proximal TCR signaling events

targets such as LAT and Slp-76. This ultimately leads to the activation of the transcription factors NFAT, NFκB and AP-1 and the subsequent induction of specific gene transcription leading to cellular proliferation, differentiation and activation. CD45 is required for this pathway, as Lck is activated by dephosphorylation by CD45. Dephosphorylation of Lck by CD45 is obligatory for activation of Lck kinase activity.

Regulation of Lck

Lck is regulated by balanced phosphorylation and dephosphorylation of two regulatory tyrosine residues (figure 3). In the mouse these residues are Y_{394} and Y_{505}. Y_{394} is located within the catalytic site, and its phosphorylation leads to activation of the Lck kinase activity. Y_{505} is known as the negative regulatory site. When it is phosphorylated, it interacts with an intramolecular SH2 domain, creating an inactive conformation through occlusion of the kinase active site.

The protein tyrosine phosphatase CD45 plays an integral role in the regulation of Lck phosphorylation and activity. This was first shown using CD45-deficient cell lines where it was observed that in the absence of CD45, Lck was hyperphosphorylated on the negative regulatory tyrosine (Y_{505}) in CD45 deficient cell lines (80). Further studies showed that these CD45-deficient T cell lines were impaired in their ability to respond to antigen (16). CD45 has thus been implicated in the dephosphorylation of the negative regulatory residue of Lck, which in turn leads to partial activation of Lck (16, 55, 80). This is only the first step in Lck activation. Full activation requires that the positive regulatory site,

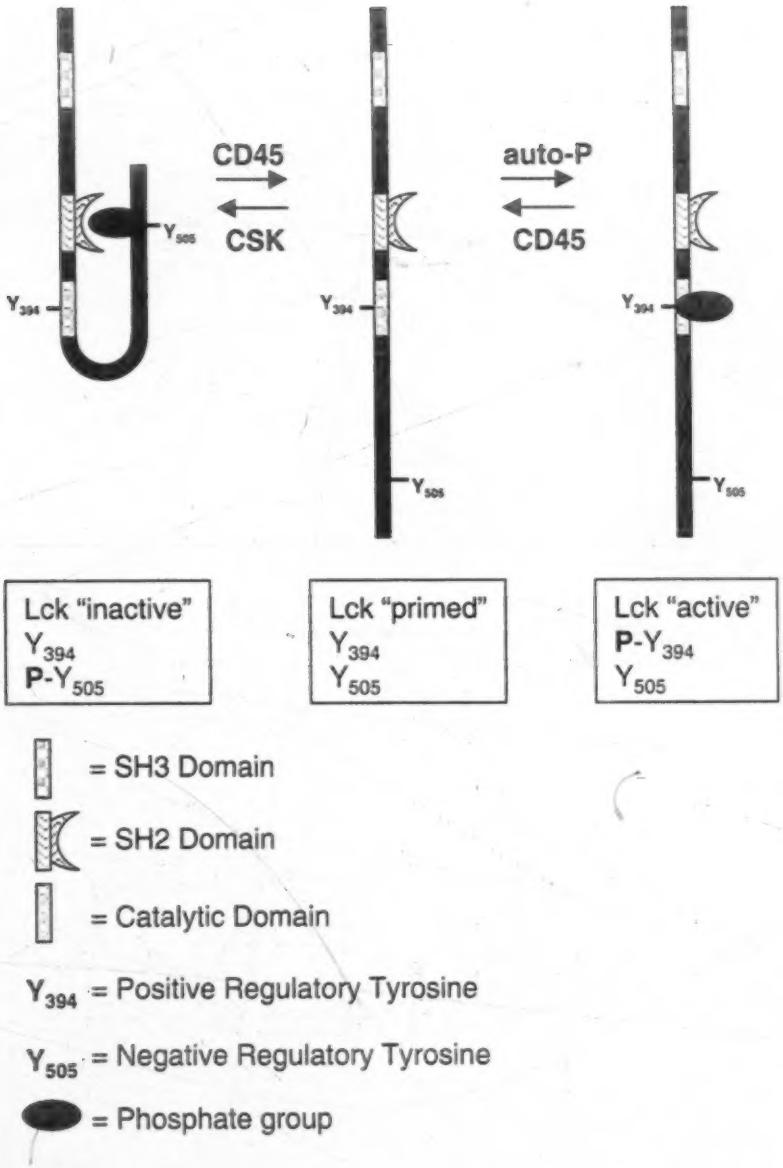


Figure 3: Regulation of Lck by Phosphorylation and Dephosphorylation

Y_{394}, also becomes phosphorylated, a step that is accomplished through trans- or auto-phosphorylation. Another level of complexity is added to the CD45-Lck relationship, in that CD45 has been implicated in the dephosphorylation of Y_{394} as well as Y_{505} (28), so that the role of CD45 in Lck regulation may be to maintain Lxk in a "primed" state, not fully active, but instead in a state of readiness for activation.

The kinase responsible for phosphorylation of Y_{505} is C-terminal Src-family kinase (Csk) (106). The regulation of Csk suggests a role for localization or cellular compartmentalization in the control of Lck in T cell membrane-proximal signaling. Csk is found in the proximity of Lck and the TCR complex only in resting cells (106). Upon activation, Csk is released from this region of signaling, allowing the phosphatase activity of CD45 to dominate, as at least a small population of cellular CD45 is likely to remain in this region (30). Dephosphorylation of Y_{505} by CD45 then leads to partial activation of Lck .

$Lck^{-/-}$ Thymic Development Phenotype

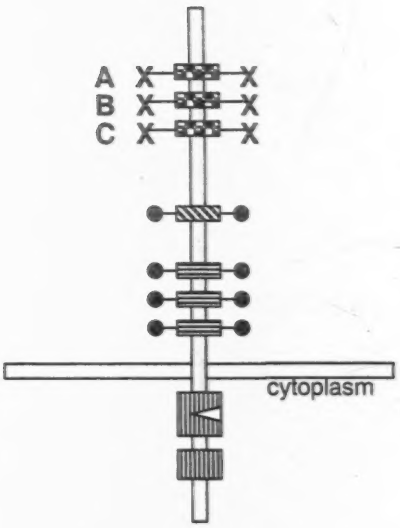
$Lck^{-/-}$ mice display a block in thymocyte development at the DN3 stage. The numbers of DP thymocytes are greatly reduced, mature SP thymocytes cannot be detected and there are only very few peripheral T cells (72). The same block is seen in TCRβ mutants, RAG-null, SCID and pTα mutants. All of these mice, including $Lck^{-/-}$ are deficient in signaling through the pre-TCR, either at the level of presentation of the pre-TCR complex or in the signaling steps immediately downstream of pre-TCR ligation.

CD45

CD45 is a receptor-type transmembrane protein tyrosine phosphatase that is one of the most abundantly expressed cell surface glycoproteins on nucleated hematopoietic cells (111) (figure 4). It ranges in size from 180kDa to 240kDa, and this variability in size is due to alternative splicing of three exons corresponding to the membrane-distal regions of the extracellular domain designated A, B, and C (encoded by exons 4, 5, and 6 respectively). The cytoplasmic domain of CD45 contains two PTPase homology domains, designated D1 and D2. The D1 phosphatase domain is sufficient to rescue TCR signalling in a CD45-deficient cell line, while D2 does not have phosphatase activity, and its exact function is unclear. It has been suggested that D2 may be involved in physical interactions that contribute to stability or regulation of function of CD45 (27).

CD45-deficient Cell Lines

The involvement of CD45 in the control of TCR signaling has been shown using CD45-deficient cell lines. It has been observed that cell lines lacking CD45 are defective in their ability to signal through the TCR (61, 82). This defect can be at least partially reversed through the expression of enzymatically active CD45 (60, 61, 114). However, this approach carries with it some intrinsic problems. The genomic organization of CD45 is unusual in that there is no tissue specific promotor, and even more unusual is the fact that there is no



= Alternatively Spliced Region

= Cysteine-Rich Domain

= FibronectinIII-like Repeats

= Phosphatase Homology Domains

= N-linked glycan

= O-linked glycan

Figure 4: The Domain Structure of CD45

traditional TATA box within 2.5 kb of exon 1 (50). Transfection of Jurkat cells with CD45 cDNA was able to restore TCR-mediated signal transduction to some degree, but not to wildtype levels. Also, expression of CD45 was only 30% of wild type levels (60). Recently, minigene constructs have been used in an effort to unravel the mysteries of CD45 gene regulation (114). These constructs have revealed that regions of both the 3' and 5' UTRs , along with some intronic sequences are required to increase expression levels and achieve tissue specific expression. However, even with the minigene construct it has not been possible to achieve endogenous levels of expression.

CD45 Knockout Mice

Three independent groups have developed CD45-deficient mouse models, all of which display profound blocks in thymocyte development and abnormal antigen receptor-mediated signaling. The first of these mouse lines was originally developed in order to examine the function of the alternative splicing of CD45, therefore it was generated by introducing a disruption in CD45 exon-6, which encodes one of the variably-spliced regions of the external domain (59). Somewhat surprisingly, these CD45-exon6$^{-/-}$ mice lack CD45 expression on all but a small subset of mature thymocytes and peripheral T cells, with the expressed CD45 probably resulting from the splicing out of the mutated exon (59). Peripheral T cells from these mice were tested for their ability to proliferate in response to both TCR cross-linking and PMA + calcium ionophore stimulation, and were found to be deficient in both responses. The T cells that do express

CD45 were purified and tested for their ability to proliferate in response to these same conditions, and were also found to be deficient in their response (59). Antiviral CTL responses *in vivo* were also completely abrogated in these mice (59).

The second CD45-deficient mouse line to be developed completely lacked CD45 expression, thus representing the first complete knockout of the CD45 gene (15). This CD45-null mouse line was created through a targeted disruption of exon 9, which is common to all CD45 isoforms The number of peripheral T cells found in these mice was typically reduced by at least 10-fold with respect to wild-type littermate controls, and the T cells that were present were profoundly impaired in their ability to respond to activation-inducing signals (15). These same thymocytes were also deficient in their ability to respond to TCR-mediated apoptotic signals in fetal thymic organ culture (FTOC) (15).

A third CD45-deficient mouse line was generated, this time with an interruption in exon 12 (67). By FACS, these mice do not express detectable levels of cell surface CD45 on any cells, although a small amount (approximately 5% of WT levels) of a non-membrane associated truncated variant of CD45 could be detected cytoplasmically (67). The thymic development phenotype of these mice is very similar to the phenotype of both the CD45-null (exon-$9^{-/-}$) and CD45 exon$6^{-/-}$ mice, strongly supporting a central role for CD45 in thymic development, both at the DN→DP transition and at the DP→SP transition.

CD45 Knock-Out Mice: Thymic Developmental Phenotype

All of the CD45$^{-/-}$ mouse models examined display profound defects in thymocyte development (15, 59, 67). The defects observed in the three lines were very similar, although more pronounced in the CD45-null and CD45-exon12$^{-/-}$ lines, potentially because of the low levels of CD45 expression that is still present in the CD45 exon6$^{-/-}$ mice. Evidence of impaired thymocyte development is observed at two distinct stages in CD45-deficient mouse lines. There is an initial, and relatively minor block at the DN→DP transition, and then a much more profound block at the DP→SP transition (15, 59, 67).

The progression of thymocyte development is studied by isolating thymocytes from the thymus of wild-type or mutant animals and comparing the FACS staining profiles for relevant cell surface molecules. If thymocyte development is blocked, a particular thymic developmental subset will accumulate. Thymocytes from CD45-null mice were stained for CD4 and CD8, and an increase in the number of DN thymocytes was observed, along with a decrease in the number of DP thymocytes. Comparison of the relative numbers of the various thymic populations revealed that progression through the stages of thymocyte development was abnormal in the absence of CD45. The DN:DP ratio in CD45-null (exon9$^{-/-}$) mice was 1:2, while wild-type littermates displayed a ratio of 1:8, indicating that there is an decreased ability of DN cells to progress to the DP stage in the absence of CD45 expression (15). DN thymocytes were further stained for CD44 and CD25 to elucidate the specific DN block. There was an increase in the CD44^{-}/CD25^{+} (DN3) population, together with a decrease in the

$CD44^-/CD25^-$ (DN4) population, indicating a block at the DN3 stage (15). Progression beyond the DN3 stage requires transmission of signals through the pre-TCR complex through classical membrane-proximal pathways. Thus, the DN3 block is not at all surprising in the absence of CD45, as it is well known that CD45 is required for signaling through the TCR, and specifically for the activation of Lck (16). Thymocytes from the CD45-null line exhibit dysregulation of other major membrane-proximal players in TCR-mediated signaling, including Fyn, TCRζ and ZAP-70 (103).

Interestingly, however, the CD4/CD8 stain revealed that a substantial subset of CD45-null thymocytes was capable of progression beyond the DN3 stage, and that within this subset, a further block was observed at the DP→SP transition. In CD45-null mice, the DP:$CD4^+$SP ratio was 18:1, compared with 4:1 in their wild-type littermates (15). The absolute numbers of SP thymocytes is also reduced in these mice, as CD45-null mice have only approximately 10% of the $CD4^+$SP thymocytes as wild-type littermate controls (15). The critical DP→SP transitional step is controlled by positive and negative selection, processes which depend heavily on the avidity of interactions that occur between cells, and in particular the interactions that occur between the TCR and MHC/peptide complexes. CD45 is expected to play a role in TCR signaling through its ability to activate Lck. However, the fact that CD45-null mice display a different thymic developmental phenotype than the $Lck^{-/-}$ mice, in that $Lck^{-/-}$ mice display a block at the DN3 stage while $CD45^{-/-}$ mice are capable of

development to the DP stage, suggests the possibility that CD45 may play another as yet unknown role in thymocyte development.

CD45 Knockout Mice: Mature T Cells

CD45-deficient mice have greatly decreased numbers of mature T cells. In the CD45-null line there is a 10-fold reduction in $CD4^+$SP splenocytes and at least a 4-fold reduction in $CD8^+$SP splenocytes compared to wild type littermate controls (15). The few peripheral T cells that are found in these mice often express TCR with high avidity for self-peptide/MHC complexes, and as such have an unusually high potential for autoreactivity (108).

CD45: Positive Or Negative Selection?

The DP→SP transition encompasses the processes of positive and negative selection, both of which involve signals through the TCR. CD45 knock-out models alone are not sufficient to determine which of these processes requires CD45. The CD45 $exon6^{-/-}$ and CD45-null mouse models, although they clearly showed the importance of CD45 in thymic development, were unable to sufficiently address the question of whether CD45 was important for positive or for negative selection.

During thymic development, a strong signal presented through the TCR can signal apoptosis, or negative selection. This strong signal can be delivered in fetal thymic organ culture (FTOC) of thymi through the addition of anti-CD3ε mAb (99). Treatment of FTOC from CD45-null mice with anti-CD3 revealed that

these thymocytes do not respond to apoptotic signals presented through the TCR. $CD45^{+/+}$ FTOC responded to anti-CD3ε antibodies with an increase in DNA fragmentation, indicative of an increase in apoptosis, while CD45-null FTOC did not increase levels of apoptosis above basal levels at all in response to the same treatment (15). There was no difference in the ability of other apoptotic stimuli, such as ionomycin, dexamethasone and Fas ligand to induce apoptosis in $CD45^{+/+}$ or $CD45^{-/-}$ FTOC (15). This defective apoptotic response implicates CD45 in negative selection, but does not address the involvement of CD45 in the process of positive selection. Defective positive selection is suggested by the phenotype of CD45-deficient mice, as the number of SP cells is dramatically reduced.

In apparent contradiction to the defective anti-TCR apoptotic pathway observed in CD45-null thymocytes, CD45 $exon6^{-/-}$ mice were able to specifically delete thymocytes bearing superantigen-reactive TCRs (59). There are at least two potential explanations for this finding. First, it may be explained by the fact that CD45 $exon6^{-/-}$ mice still express low levels of CD45, and that this may be enough to mediate signaling in response to superantigen crosslinking. Another explanation, which seems to be supported by subsequent findings in the CD45 $exon12^{-/-}$ mice is that negative selection is not ablated in the absence of CD45, but that the threshold required to activate negative selection is considerably higher. It may be that the interaction between the superantigen and its specific TCR is strong enough to reach the increased threshold imposed by the lack of CD45 expression.

The role of CD45 in positive and negative selection was explored further using CD45 exon12$^{-/-}$ mice (67). It was demonstrated that within the DP population in these mice there was a decrease in the number of CD69^{+} thymocytes, which is consistent with a block in positive selection. Also, the possibility that the defects in thymocyte development were due to lack of CD45 expression on cells other than thymocytes (such as dendritic cells and macrophages) within the thymus was examined. CD45-deficient thymocytes were used to reconstitute irradiated WT mice, and there was no difference in development from that observed on a CD45-deficient background indicating that the developmental abnormalities are due to factors intrinsic to the thymocytes (67).

A closer examination of positive and negative selection was afforded by the introduction of various transgenic TCRs onto the CD45 exon12$^{-/-}$ background along with RAG$^{-/-}$ mutation to prevent endogenous TCR rearrangement. The results of these studies revealed that, while CD45 is not absolutely required for positive and negative selection, the signal threshold required to achieve both is increased when CD45 is not expressed (67). This is true for TCRs with specificity for both MHCI and MHCII (67). Thus it can be concluded that CD45 is important for the signals transmitted through the TCR for both positive and negative thymoctye selection.

These findings bring to light an important caveat that should be kept in mind regarding studies of positive and negative selection. It is necessary to use TCR transgenic mouse models in order to follow the development of thymocytes

in response to specific ligands. Within the physiological thymic environment, there are numerous different TCRs that are subjected to selection processes simultaneously. It is difficult to say whether or not the observations made of the development of a few chosen TCRs in a greatly simplified microenvironment can be extrapolated to provide assumptions that reflect physiological selection in any way. However, this system is useful for looking at molecular interactions that may be important during development, and combining knock-out models such as the CD45-deficient systems with TCR transgenics can provide useful models to start to examine which molecular interactions may be important in the dauntingly complex process of thymocyte development.

Expression Of Constitutively Active Lck In CD45-null Mice

As the CD45-deficient mouse models do display at least a partial block at the same developmental stage as the $Lck^{-/-}$ mouse, the question arises as to whether or not the block in the CD45-deficient mice is caused by the inability of Lck to become activated. In order to investigate this question a constitutively active form of Lck (Lck Y505F) was expressed in CD45-exon$6^{-/-}$ mice under the control of the Lck proximal promoter (90). This Lck mutant is constitutively active because it cannot be phosphorylated at the negative regulatory site (Y_{505}) and thus cannot be downregulated in that manner. Constitutively active Lck, when expressed on its own, results in a highly abnormal thymic development phenotype. Development progresses to the early DP stage, in that thymocytes do express both CD4 and CD8. However, there is a failure to rearrange the

TCRβ chain and to upregulate CD3 expression, both processes that normally occur in the DN stage (1). These abnormalities are presumably due to premature signaling, telling the cell that a productive β-chain rearrangement has occurred. Since signals through a successfully rearranged β-chain result in the termination of further rearrangements of the β chain, the signals generated by the presence of constitutively active Lck at the early stages of thymocyte development can explain the lack of TCRβ chain rearrangement. In order to bypass this problem the Lck Y505F variant was expressed along with the DO11.10 TCR, which is specific for I-E^d presenting OVA peptide 323-329, thus providing a productively rearranged β-chain and effectively avoiding the early DP developmental block (5). When Lck Y505F is expressed in the CD45 exon-$6^{-/-}$ mice along with the DO11.10 TCR, the number of CD4SP thymocytes is very similar to the number found in normal mice, suggesting that constitutively active Lck can overcome the developmental block imposed by CD45 deficiency. Expression of the DO11.10 TCR alone did not result in successful development of CD4SP thymocytes (90).

The expression of the Lck Y505F and the DO11.10 TCR on the CD45-exon$6^{-/-}$ was also able to restore antigen-induced apoptosis. In normal mice, the injection of OVA peptide 323-329, but not OVA peptide 324-334, results in decreased numbers of live thymocytes expressing the DO11.10 TCR, as well as an increase in apoptosis of the remaining thymocytes, as evidenced by an increase in annexin V staining. There was no increase in apoptosis of thymocytes from CD45-exon$6^{-/-}$, DO11.10 TCR transgenic mice (with no Lck Y505F) in response to either peptide, while the expression of Lck Y505F resulted

in both decreased TCR expression and increased annexin V staining, similar to that seen in thymocytes with a functional CD45 allele (90). This result suggests that, at least for this particular TCR, Y505F Lck is sufficient to generate signals required to restore negative selection in the absence of CD45.

Taken together, the results from the Y505F Lck/CD45 exon6$^{-/-}$ mouse model suggest that constitutively active Lck can restore thymocyte development in the absence of CD45. It is important to keep in mind, however, that this model is a very artificial system. Lck is constitutively active, and is therefore not subjected to the usual regulation by CD45 and Csk. If thymocyte development is indeed the product of numerous intracellularly integrated signals, the constitutive activity of Lck may be sufficient to provide enough signal to reach the threshold of signal strength required to trigger positive and negative thymic selection events. Results obtained from the Y505F Lck/CD45$^{-/-}$ model are not inconsistent with a model that includes CD45 as an important contributor to selection decisions in the presence of wild type Lck, only that the contribution of CD45 is not required in the presence of constitutively active Lck.

CD45 Extracellular Domain

Despite more than a decade of research, the function of the external domain of CD45 remains a mystery. When it was discovered that there is a high degree of homology between the tandemly-repeated cytoplasmic domains of CD45 and PTP-1B (20), and that CD45 does indeed have phosphatase activity (107), it seemed that it would follow that CD45 would behave as a cell surface

receptor. Thus it was proposed that CD45 activity would be modulated by the binding of a specific ligand, in a manner similar to the modulation of receptor protein tyrosine kinases (PTKs) such as the epidermal growth factor receptor (EGFR) (54). This idea was particularly attractive, as PTKs generally transmit positive signals, such as signals promoting growth and proliferation. CD45 could act to oppose these positive signals, in essence contributing to a regulatory balance between phosphorylation and dephosphorylation. Research over the past fifteen years has, however, revealed that the role of CD45 in the regulation of tyrosine phosphorylation is much more complex. Firstly, we have seen that dephosphorylation by CD45 does not always act as a negative signal, and in fact CD45 acts as an essential positive regulator for T cell signaling. Secondly, if there is a specific ligand for CD45 it has not yet been discovered. Numerous attempts have nevertheless been made to determine the function of the extensive and very complex extracellular domain of CD45.

CD45 Isoform Expression

CD45 possesses three variable exons within the genetic region encoding the external domain, resulting in the potential for eight distinct CD45 isoforms. The use of the variable exons results in significant variability in the size and carbohydrate content of the external domain. The expression of the various isoforms of CD45 is highly regulated, and seems to be dependent upon developmental stage, activation state and/or cell type (51, 111). CD45 lacking all of the variable regions is known as CD45R(0), and isoforms of CD45 including

exons 4, 5, or 6 are known as CD45R(A), (B) or (C), respectively (fig. 4). Electron microscopy studies revealed that the external domain of CD45R(0) extends approximately 28nm from the surface of the cell and CD45R(ABC) extends 51nm (66). To put this into perspective, the TCR only extends 10nm from the cell surface (95). The size, regulation and abundance of the CD45 external domain suggest some particular importance for this domain. However, what should be simple studies of isoform expression have been hampered by a number of complicating factors (111). First, in the mouse there is no antibody specific for the CD45R(0) isoform. Second, many of the antibodies that are available depend upon glycosylation patterns for recognition, and as such recognition may not be the same in different cells with slightly different patterns of glycosylation. Third, antibodies developed for a particular isoform may have the capability to recognize more than one isoform. For instance, an antibody specific for CD45R(A) may be able to recognize CD45R(A) as well as CD45R(AB). As if the inherent problems with the antibodies were not enough to make this area particularly confusing, most cells express more than one isoform of CD45 simultaneously, and CD45 isoform expression changes as a cell undergoes differentiation. The area of CD45 isoform expression is so confusing, in fact, that an article entitled "Confusion Over CD45 Isoform" was published in Nature in 1991 (68).

In spite of all of these problems, a general expression pattern for the isoforms of CD45 has emerged. Most of this work was done using human thymocytes. Thymic progenitors (47) and a percentage of early DN thymocytes

(37, 64) are positive for expression of the variable exons (CD45R). The majority of thymocytes, however, express the low molecular weight CD45R(0) isoform (57), and stimulation of immature thymocytes expressing the variable regions results in a switch to CD45R(0) expression followed by apoptosis (69). Histological studies have shown that CD45R expression is higher in the thymic medulla than it is in the cortex, leading to the suggestion that perhaps expression of the variable exons is upregulated following positive selection (43). However, $CD45R(0)^{+}$, $CD45R(A)^{-}$ thymocytes that have apparently already undergone positive selection have been described, suggesting that positive selection may not be the only step required to induce expression of the variable regions (78). Other studies have shown a potential link between selection events and the expression of CD45R isoforms, as an increase in both CD45R(B) and CD45R(A) was observed in thymocytes undergoing positive and negative selection events (118).

Recently CD45 isoform expression was studied using a combination of RT-PCR and four-colour flow cytometry (38). These studies show that the various isoforms of CD45 are indeed tightly regulated throughout the course of thymocyte development, and that the pattern of expression of these isoforms is very complex. Without an understanding of the function of the external domain of CD45, or even whether or not there is a ligand makes it extremely difficult to ascribe any functional significance to the findings of these various studies of CD45 isoform usage. For the time being, it is safe to say that the pattern of expression of the CD45 variable exons is constantly changing in a

developmentally regulated manner. The complexity and variability of these expression patterns, along with the confounding factor that each T cell or thymocyte is capable of expressing various isoforms leads to the lack of clarity that is evident in this area at this time.

CD45 Glycosylation

The extracellular domain of CD45 contains numerous sites for both N- and O-linked glycosylation, with the O-linked glycosylation sites being found largely within the variable exons. O-linked carbohydrate regions form rigid rod structures due to the presence of numerous α-helix-breaking residues and lack of cysteine residues. Also, these O-linked glycosylation sites can be modified by sialic acid, resulting in substantial differences in size, carbohydrate content, shape and negative charge among the various CD45 isoforms. Since the O-linked carbohydrate modification sites are found within the differentially spliced regions, usage of these exons can result in dramatic differences in the overall structure of the extracellular domain (39, 88). Carbohydrate modification of the extracellular domain is extensive, as evidenced by deglycosylation studies where the two membrane-proximal domains from the extracellular region of CD45 were expressed and then subjected to PNGaseF (peptide:N-glycosidaseF) treatment to remove carbohydrate. PNGaseF treatment resulted in a decrease in molecular weight from between 43-67kDa for this portion of the protein down to 20-23kDa, which is consistent with the expected molecular weight of the peptide backbone of these domains (105).

The Search for a CD45 Ligand

There are two generally accepted requirements for a ligand: first, the molecule must bind specifically to its receptor, and second, this binding must evoke a physiological response by the receptor molecule (111). In light of these requirements, the extensive search for a CD45 ligand has not been successful, although some molecules that are capable of binding to the extracellular domain of CD45 have been discovered.

The first such potential CD45 ligand to be reported was CD22 (8, 102). CD22 is a B-cell glycoprotein with significant homology to several adhesion molecules, such as neural cell adhesion molecule (NCAM) (24) and the vascular adhesion molecule V-CAM/In-CAM110 (79). A soluble form of CD22 (CD22Rg) was generated, and was found to interact with CD45. This interaction could reportedly be inhibited by the UCHL-1 antibody (anti-CD45R0), suggesting that this interaction was indeed a specific interaction between CD45 and CD22 (102). Also, the presence of CD22Rg could block calcium flux in response to anti-CD3, and result in a decrease in phosphorylation level of the CD45 substrate PLCγ1 (8). A short while later, however, another group showed that the antibody UCHL-1 actually did not block CD22 adhesion with T- or B-cells, and also that CD22 adhesion can be blocked by a component present in some ascites fluid (32), providing a possible explanation for the earlier report of UCHL-1 blocking (102). Finally, it was determined that CD22 does not exclusively nor specifically bind CD45, but that CD22 is a sialic acid-binding lectin with specificity for α2-6 sialic

acids, which are found on numerous cell surface glycoproteins (93). Since it had now been shown that CD22 did not specifically bind to CD45, the fact that CD22Rg was able to block anti-CD3 mediated T-cell responses could no longer be interpreted to mean that the CD22/CD45 interaction was modulating that response. Instead, CD22 is capable of binding numerous sialic acid-containing cell surface glycoproteins, any of which may be important in the mediation of T-cell responses (111).

Galectin-1, a molecule expressed in the thymus by thymic epithelial cells, has been implicated in CD45 binding as well (119). This CD45-galectin-1 interaction may be important for apoptosis induction in immature $CD3^{-/-}$ or $CD3^{lo}$ thymocytes during positive and negative selection (81). Galectin-1 binding, like CD22 binding however, is not specific for CD45. In fact, CD45, CD43, CD7, CD4, and CD3 were all shown to be able to bind to galectin-1, and no direct evidence was presented to show that the CD45/galectin-1 interaction was required for the induction of apoptosis (76). It was shown that the $CD3^{-}CD4^{-}CD8^{-}$ ARR cell line is susceptible to galectin-1-induced apoptosis (81), suggesting that at least one of CD45, CD43 and CD7 is involved in the induction of apoptosis by galectin-1. It was also shown that galectin-1 binding induces the re-organization of CD45, CD43 and CD7 on the cell surface, but there is no evidence to show that the initial triggering event for apoptosis in these cells is galectin-1 binding to CD45.

Another molecule found to be capable of interacting with CD45 is glucosidase II (GII) (7). GII functions in carbohydrate processing within the

endoplasmic reticulum, where it is central to the protein folding process. The interaction between CD45 and GII displays characteristics of a mannose-based lectin interaction, as it can be inhibited by the addition of exogenous mannose, and removal of glycans from CD45 by endoglycosidaseF treatment abrogates the interaction (10).

Numerous other studies have been done to try to determine the specific ligand for CD45, but these have also proved to be unsuccessful. The external domain was expressed as a soluble secreted glycoprotein, but this recombinant protein did not show any significant effect on lymphocyte adhesion, proliferation or cytolysis. Furthermore, this protein failed to bind specifically to lymphoid cells (110, 120). However, CD45 external domain interactions may require highly specific carbohydrate modifications, which simply may not be present in a recombinant protein expression system.

Another possible function for the CD45 external domain is the interaction with other molecules on the same cell surface. There are a number of techniques available for studying this type of interaction, including chemical cross-linking, co-capping and fluorescence resonance energy transfer (FRET). The results from these studies are very disappointing, as even under nearly identical conditions in some cases completely different results were obtained (111). It is important to keep in mind, however, that CD45 comprises nearly 10% of total surface protein, making it very difficult to separate cross-linking or co-capping that occurs due to close proximity of the partners from actual physical associations.

Chimeric Protein Studies

The expression of chimeric proteins that include or do not include the domain in question is one way to approach the determination of domain function. A number of such studies have been done with the CD45 external domain, with some rather unexpected, and perhaps even troubling results. Three separate groups made CD45 chimeric proteins, each with a completely different external domain. One group used the EGFR external domain (26), another used MHCI external domain (52), and a third group used nothing but a short N-terminal sequence from $p60^{c\text{-}Src}$ (115). All three groups showed that TCR-mediated signaling capability could be restored, at least to some extent, in CD45-deficient cell lines in the presence of these CD45 chimeras lacking the natural CD45 external domain. Recently, however, it has been suggested that the CD45 external domain may in fact be required for the correct subcellular localization of CD45 (56), and that the external domains used on the earlier CD45 chimeras may have still been able to mediate the required localization of CD45.

DC-SIGN

Lectins are cell surface or soluble proteins that bind sugar ligands presented on specific proteins. Binding in C-type lectins is mediated by the carbohydrate recognition domain, or CRD. Within the CRD is usually a calcium-binding site, as lectin interactions are often calcium dependent. One of the best characterized of these is DC-SIGN (Dendritic cell-specific, ICAM-3 grabbing,

non-integrin). DC-SIGN is a transmembrane protein with an extracellular C-type lectin domain that specifically binds mannose. It has recently been shown to mediate adhesion between T cells and DC in what appears to be an antigen-independent manner. It has been proposed that DC-SIGN, through an interaction with ICAM-3, mediates early loose adhesion between DC and resting T cells (41). In this model, this early antigen-independent adhesion would hold the cells in close contact in order to allow the TCR to sample the surface of the DC for activating MHC-peptide complexes. Once the T cell locates the relevant ligand it becomes activated and begins to express its own adhesion molecules so that the DC-SIGN-mediated adhesion is no longer required on active T cells (41).

INTEGRINS

Integrins are cell surface receptors that can mediate adhesion both between cells and between cells and the extracellular matrix (ECM). Both types of interactions have been shown to be important during thymocyte development (4). Interactions between LFA-1 (leukocyte functional antigen-1) and ICAM-1 (intercellular adhesion molecule-1) have been shown to play an important role in the maturation of thymocytes from DN→DP stages (35). Also, expression and glycosylation patterns of the VLA (very late antigen) family of integrins are developmentally regulated during thymocyte development (116, 117). VLA-4, which is comprised of the integrin chains $\alpha_4\beta_1$, is expressed on a subset of immature DP thymocytes can bind to both VCAM-1 (Vascular Cell Adhesion Molecule-1) or FN (fibronectin) (31), both of which are expressed in the thymus.

However, VCAM-1 is expressed primarily by thymic epithelial cells of the cortex and corticomedullary junction, while FN is expressed mainly in the medulla. Furthermore, it has been demonstrated that adhesion between thymocytes and cultured human thymic epithelial cells is mediated by VLA-4/VCAM-1, as adhesion between these cell types can be inhibited by both anti-VLA-4 and anti-VCAM-1 mAbs (86). These findings suggest that VLA-4/VCAM-1-mediated adhesion could play an important role in the essential interactions that occur between thymocytes and the thymic stromal microenvironment during thymocyte development.

CD45 Regulation of Integrin-Mediated Adhesion

While the function of CD45 has been extensively studied in both T- and B-cells, its role in other leukocytes is much less clearly defined. Some work done in macrophages, however, implicates CD45 in the regulation of integrin-mediated adhesion (84). Subsequent studies in T cells lines have supported these findings (96). Because Src-family kinases are important for regulation of integrin-mediated adhesion (22), and due to the known role of CD45 in the regulation of Src-family kinases (80), it was postulated that CD45 may be involved in the regulation of adhesion (84).

Primary macrophages from CD45 exon6$^{-/-}$ mice were first used to examine this possibility. As expected, the Src-family kinases Hck and Fyn were hyperphosphorylated at the negative regulatory sites in the absence of CD45. However, these kinases also displayed increased activity, the opposite of what is

found in T cells (16). Integrin-mediated adhesion in the CD45 exon6$^{-/-}$ macrophages was abnormal, in that compared to macrophages from CD45$^{+/+}$ mice they displayed a much faster rate of adhesion to tissue culture treated plates but were deficient in the maintenance of that adhesion as compared to wild type macrophages (84). Similar results were seen in T cell lines, where lack of CD45 expression correlates with enhanced adhesion to fibronectin through VLA-5 ($\alpha_5\beta_1$ integrin) (96).

The observed abnormalities in integrin-mediated adhesion in CD45$^{-/-}$ macrophages suggest that CD45 is involved in the regulation of integrin-mediated adhesion, and that at least in macrophages this is accomplished through the regulation of Src-family kinases, although the balance of this regulation may be different than that seen for antigen receptor signaling in T lymphocytes. The activation and function of Src-family kinases is likely very different at sites of adhesion than what we know of Src activity in antigen receptor-related signaling. Most notably, CD45 does not seem to be required for Src activation at sites of adhesion, instead activation is a result of interactions that occur within the adhesion site. This has been demonstrated in cell lines, as focal adhesion kinase (FAK) binding to integrins at the adhesion site can result in activation of Src-family kinases (89). The role of CD45 in macrophages may be to downregulate Src kinase activity after it has been activated by adhesion. In the absence of this regulation the signals required to maintain adhesion are lost, resulting in a dysregulation of the integrin-mediated adhesion pathway.

The fact that in the CD45 exon6$^{-/-}$ macrophages, the negative regulatory site of the Src family kinases is hyperphosphorylated, while the activity of these kinases is also increased may present some confusion with respect to the known regulation of Src-family kinases. Usually, hyperphosphorylation at this site results in a decrease in activity. However it is possible to explain how activity could be increased. It is important to remember that CD45 and the Src-family kinases are not the only proteins present in the cells that are being studied. It is true that the intracellular SH2 domain of Src kinases can interact with the phosphorylated negative regulatory tyrosine. However, there is no reason why that same phosphorylated tyrosine cannot interact with higher affinity SH2 domains found in other proteins. This would allow the active site of the Src-family kinases to remain open and accessible even in the presence of a phosphoryated negative regulatory site. In this situation then, the balance of CD45 activity could shift to dephosphorylation of the now accessible positive regulatory tyrosine, resulting in CD45 downregulating the Src kinases instead of participating in their activation. The function of CD45 observed in macrophages in the regulation of integrin-mediated adhesion illustrates how complex and delicate the balance of signaling in response to CD45 can be.

Summary

In summary, it has been shown that CD45 is required for proper development of a mature T cell repertoire, but the specific role played by CD45 has not yet been elucidated. Thymic development is dependent upon

MHC/peptide-TCR interactions, but is believed to also depend upon the avidity of interactions between thymic stromal cells and the developing thymocytes. This avidity could potentially be mediated by many of the other surface molecules found on the cells within the thymus. As the function of the abundantly expressed CD45 external domain is currently unknown, it is intriguing to consider the possibility that it may function to mediate adhesion between thymic stromal cells and developing thymocytes, and in this way contribute to the avidity of interactions between these two cell types. CD45 could also play a less direct role in the thymic selection process, in that CD45 could be involved in the regulation of adhesion molecules such as integrins.

Rationale and Hypothesis

CD45 plays a central role in the regulation of both the development and function of T lymphocytes. In our laboratory we made the observation that a $CD45^{+/+}$ cell line (SAKR) was able to adhere to monolayer cultures of thymic stromal cells (TSC), but that the $CD45^{-/-}$ variant of the same cell line ($SAKR/T200^{-/-}$) was unable to adhere to the same TSC cell line (Troy Baldwin, unpublished results). This observation, along with the known phenotype of CD45-deficient mice, suggested to us that CD45-dependent adhesion may play a role in the process of thymocyte development. The structure and glycosylation status of the external domain of CD45 makes it very tempting to speculate that there is a physiological binding partner for CD45, and the surface of thymic stromal cells presents a prime prospect for the location of this ligand. This, along

with the numerous studies that have found carbohydrate-dependent interactions between the extracellular domain of CD45 and lectin-like molecules led us to hypothesize that the extracellular domain of CD45 is important in mediating cell-cell interactions between thymic stromal cells and developing thymocytes during thymocyte development through carbohydrate – lectin interactions involving the glycosylation modifications on the external domain of CD45 and lectins on the surface of the stromal cells.

CHAPTER II

MATERIALS AND METHODS

Cell Lines

The murine T-lymphoma cell lines SAKR.TLS.12.1 (SAKR), BW5147 (BW), and the CD45-negative variants (SAKR/T200$^{-/-}$ and BW/T200$^{-/-}$) were obtained from Dr. R. Hyman (The Salk Institute, La Jolla, CA), and maintained in DMEM (Life Technologies, Burlington, ON) supplemented with 8% DCS and 100µg/mL penicillin-streptomycin.

Thymic stromal cells (TSC) were isolated in our laboratory from thymi of C57Bl/6 mice according to the protocol reported by Niebergs, A.C. *et al.* (77). Briefly, thymi from adult C57Bl/6 mice were removed. Connective tissue and other non-thymic tissues were removed using fine surgical instruments and then the thymi were then cut into 1-2mm pieces. These pieces were then placed into tissue culture dishes (Falcon) and cultured in RPMI 1640 (Life Technologies, Burlington ON) supplemented with 15% FCS (Hyclone, Logan, Utah), 2mM L-glutamine and 100µg/mL penicillin-streptomycin, 0.1mM non-essential amino acids an 0.1 mM sodium pyruvate. Under these conditions the adherent stromal cells migrate out of the disrupted thymic microenvironment and adhere to the tissue culture dish. These cells were maintained in culture through weekly trypsinization.

Antibodies and Reagents

The pan-specific anti-CD45 mAb, I3/2.3 has been described (109). The hybridomas producing the monoclonal antibodies MB23G2 and MB4B4 (anti-CD45RB), M1/8.9 and M1/9.8.1 (anti-CD45), PS/2 (anti-VLA-4), M17/5.2 (anti-LFA-1) were purchased from American Type Culture Collection (ATCC). Hybridomas were grown in Protein Free Hybridoma Medium-II (Life Technologies, Burlington, ON) and the mAbs purified by ammonium sulfate precipitation followed by either protein A or Protein G chromatography, if required. Fluorochrome-coupled mAbs RM4-4 (anti-CD4) and 53-6.7 (anti-CD8) were purchased from BD-PharMingen (Mississauga, ON). FITC-labelled secondary antibodies were purchased from Jackson Labs.

Lipophilic membrane dyes, PKH67 (green) and PKH26 (red), carbohydrates (glucose, mannose, lactose, laminarin, glucose-6-phosphate and mannose-6-phosphate) and FITC-labeled plant lectins (BS1-B4 recognizes terminal α-D-galactosyl residues; WGA recognizes N-acetyl-β-D-glucosyl residues; PSA recognizes terminal α-D-mannosyl residues; TGP recognizes α-L-fucose; and PNA recognizes immature unsialidated glycans) were purchased from Sigma Chemicals (St. Louis MO).

Adhesion Assay

Thymic Stromal Cells (TSC) were treated with versene (EDTA/PBS, Invitrogen) for 10 minutes to remove calcium from the cells and allow the monolayer to de-adhere from the surface of the tissue culture flask. Cell

numbers were quantified using a hemocytometer, and 20-40X10^6 each of lymphoma cells (SAKR, SAKR/T200$^{-/-}$, BW or BW/T200$^{-/-}$) and TSC were washed 3X in PBS and then stained with green PKH67 and red PKH26 lipophilic dyes, respectively. The cells were then washed in PBS supplemented with 4% DCS, quantified again using a hemocytometer, and resuspended at 1X10^6 cells/mL (TSC) or 2X10^6 cells/mL (lymphoma cells). The stained cells were incubated at 37°C for a 1-2 hours in PBS containing 4% DCS. Where indicated, both cell types were incubated with 5mM EDTA, various carbohydrates (100mM) or antibodies (10µg/mL) for 30 minutes following this incubation. The cells were then mixed by adding 1X10^5 lymphoma cells and 2X10^5 TSC, to a total volume of 200µL per reaction. The initial mixing was done on ice, and at time zero the cell mixtures were incubated at 37°C for the time periods indicated. At each time point the cell mixtures were vortexed and fixed by the addition of 300µL of 5% formaldehyde solution. Adhesion was measured by flow cytometry. The population of cells involved in TSC/lymphoma adhesion was considered to be the red/green double positive population on the FL-1 vs FL-2 dot plot, and is represented by the dots in the upper right quadrant (UR) of this dot plot. Conjugation was quantified through calculation of the percent TSC involved in conjugates, and was calculated as percent of cells that were double positive (upper right; UR) divided by total TSC [double positive (UR) + red TSC only (upper left, UL)] multiplied by 100%. From raw FACS data this calculation is carried out as follows: percent TSC conjugated = UR / (UR + UL) X 100%.

Surface Staining

Cells were washed 3X in PBS, quantified using a hemocytometer and suspended at $5X10^6$ cells/mL in PBS containing 4% DCS. $1X10^6$ cells were incubated at room temperature with 10µg/mL unlabeled primary antibody or 2µg/mL of FITC-labelled lectins for 20-30 minutes. Following this incubation, cells were washed 3X in PBS to remove excess antibody or lectin. At this point the lectin-stained cells were fixed in 300µL of 5% formaldehyde solution to await flow cytometric analysis. For the samples stained with unlabeled primary antibody, 2µL of an appropriate secondary antibody was added and the cells were incubated for an additional 20 minutes at room temperature in the dark. The cells were again washed 3X in PBS then fixed using 300µL of 5% formaldehyde solution. Surface staining was assessed using flow cytometric analysis. Intensity of staining obtained through this method was compared to background staining achieved when cells were stained with secondary antibody alone.

Thymocyte isolation

Thymi were removed from C57Bl/6 or C57Bl/6 Ptprc.tm1 (CD45 exon$9^{-/-}$) mice and placed in DMEM supplemented with 8% DCS. Connective tissue and other non-thymic tissue was removed with fine surgical instruments, and thymic tissue was homogenized. The resultant cell suspension was subjected to NH_4Cl lysis to remove erythrocytes. Thymocytes were quantified using a hemocytometer, and 20-$40X10^6$ thymocytes were washed 3X in PBS, stained

with green PKH67 dye, resuspended at $2X10^6$ cells/mL and incubated at 37°C for 1-2 hours. The labelled thymocytes were then utilized in the adhesion assay in the same manner as the lymphoma cell lines to assess their ability to form conjugates with TSC.

Statistics

Where error bars are included, they represent standard error calculated based on three independent experiments unless otherwise indicated. All statistical analyses were performed using Microsoft Excel.

CHAPTER III

RESULTS

Adhesion is observed between SAKR and cultured thymic stromal cells (TSC) only when CD45 is expressed on the surface of SAKR cells.

It had previously been observed by our lab that the thymic lymphoma cell line SAKR displayed a higher degree of adhesion to cultured thymic stromal cells (TSC) than did the CD45-deficient variant of SAKR (SAKR/T200$^{-/-}$) in co-culture experiments (Troy Baldwin, unpublished results). We wanted to further confirm this observation using a more quantitative method, so we adapted a flow cytometric adhesion assay for use with these cell types. Briefly, SAKR and TSC were labeled with green and red fluorescent membrane dyes, respectively. Then the two cell types were mixed together and allowed to interact over a time course of 30 minutes. These mixed cell populations were then analyzed by flow cytometry for the presence of complexes containing both red and green fluorescence. When we compared the adhesion between SAKR and TSC with adhesion between SAKR/T200$^{-/-}$ and TSC cells, we found that only in the SAKR/TSC adhesion pairing did a discrete double positive population appear (figure 5a). We consistently found that in adhesion trials between SAKR and TSC, up to 30% of TSC were involved in conjugates with SAKR, while only 10%-15% of TSC were involved in conjugates with SAKR/T200$^{-/-}$ (figure 5b). Surface stain using the pan-specific anti-CD45 mAb I3/2 indicate that CD45 is expressed on the surface of SAKR but not SAKR/T200$^{-/-}$ (figure 5c).

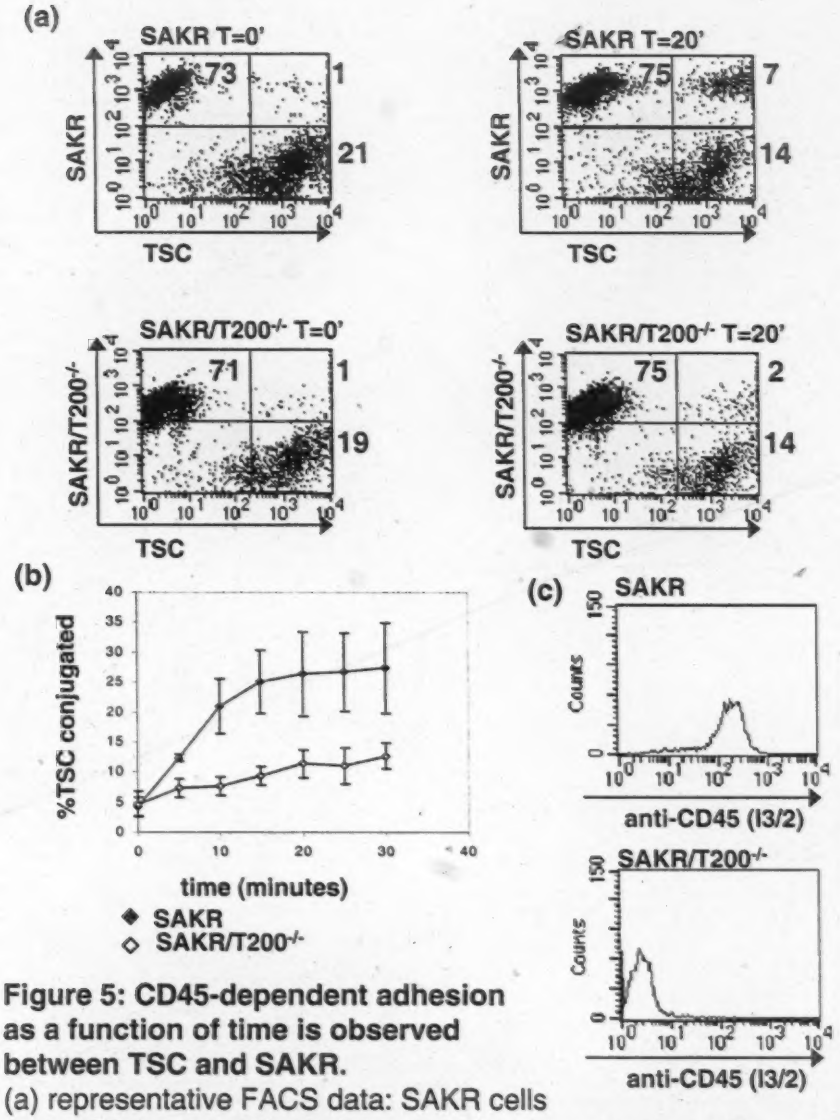


Figure 5: CD45-dependent adhesion as a function of time is observed between TSC and SAKR.

(a) representative FACS data: SAKR cells and TSC were subjected to an adhesion assay, as described. The percentage of cells appearing in each quadrant is indicated. (b) Graphical representation of part (a). Percent TSC conjugated is determined as follows: percent TSC conjugated = UR / (UR + UL) X 100%. This figure represents an average of 3 separate experiments. (c) Surface stain for CD45 using the pan-specific anti-CD45 mAb I3/2.

Characterization of cultured thymic stromal cells

The cultured thymic stromal cells used in these experiments were grown out of the thymus in a non-specific manner, simply by slicing the thymus into small pieces and then allowing the thymic stromal cells to adhere to tissue culture-treated plates. Thus the thymic stromal cells potentially represent a heterogeneous population. A similar isolation technique had been utilized previously (77) to obtain cells that had characteristics of thymic epithelial cells, so we utilized mAbs against some known thymic epithelial cell markers to see if we had isolated classical thymic epithelial cells. Surface staining with mAbs against both medullary and cortical thymic epithelia could not be detected (data not shown). However surface staining of *ex vivo* thymic stromal cell populations using the same commercial antibodies was unsuccessful (Josh Christenson, personal communication).

SAKR still adhere to TSC cultured in D-valine

There is potential that the cultured thymic stromal cells could be composed of other thymic cell types, such as fibroblasts. Epithelial cells are capable of isomerizing D-valine to L-valine, but fibroblast cells are not capable of this isomerization reaction (42). We grew and maintained some of our TSC cells in D-valine-containing DMEM, and found that although these cells grew much more slowly in the presence of D-valine, they were still viable and maintained an epithelial cell morphology (figure 6a). Also, when the ability of TSC grown in

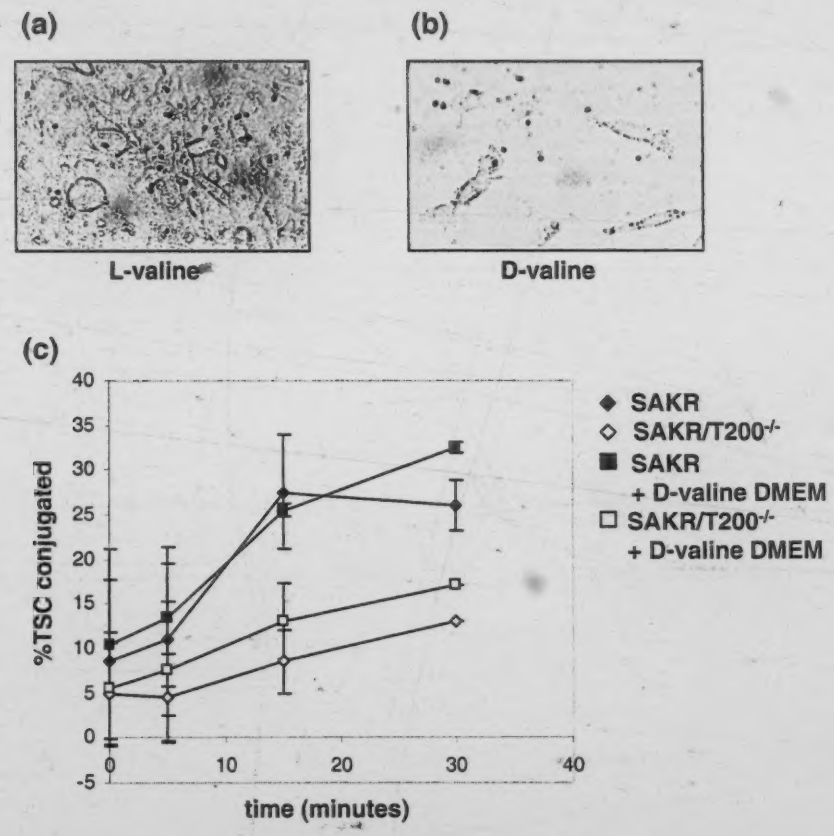


Figure 6: SAKR still adhere to TSC cultured in D-valine
(a) light micrograph of TSC grown in regular TSC media (b) light micrograph of TSC grown in TSC media with D-valine substituted for L-valine. Pictures in both (a) and (b) were taken three days after a single culture was split into two separate plates with each type of media. (c) Conjugate assay was performed as described between SAKR or SAKR/T200$^{-/-}$ cells and TSC grown either in regular TSC media or TSC media with D-valine substituted for L-valine. This figure represents and average of three separate experiments.

D-valine to form conjugates with SAKR was compared with TSC grown in regular media there was no observable difference (figure 6b). These results are consistent with the TSC being epithelial in nature, but additional studies, such as staining with fluorescently labeled cytokeratin will be required to confirm this origin.

Adhesion between SAKR and cultured TSC is cation-dependent.

As lectin-carbohydrate interactions are often dependent upon divalent cations such as Ca^{2+} and Mg^{2+}, the first step required to test our hypothesis was to determine whether or not the observed adhesion between SAKR and TSC required divalent cations. Adhesion between SAKR and TSC was measured therefore in the presence of 5mM EDTA. The lymphoma cells and the TSC were incubated with 5mM EDTA for 30 minutes following the rest period and the cells were allowed to interact in the presence of this EDTA. Following this treatment the adhesion between SAKR and TSC was decreased to levels similar to that observed between SAKR/T200$^{-/-}$ cells and TSC, where only approximately 10% of TSC were involved in conjugates with SAKR, compared with up to 30% in the absence of 5mM EDTA (figure 7a). This observation is consistent with our hypothesis as it strongly suggests that observed adhesion is indeed cation dependent. If the adhesion is indeed lectin-mediated we would expect to see this cation dependence. In order to ensure that the EDTA treatment was not having a detrimental effect on the cells, the cells were washed 3X in PBS following the

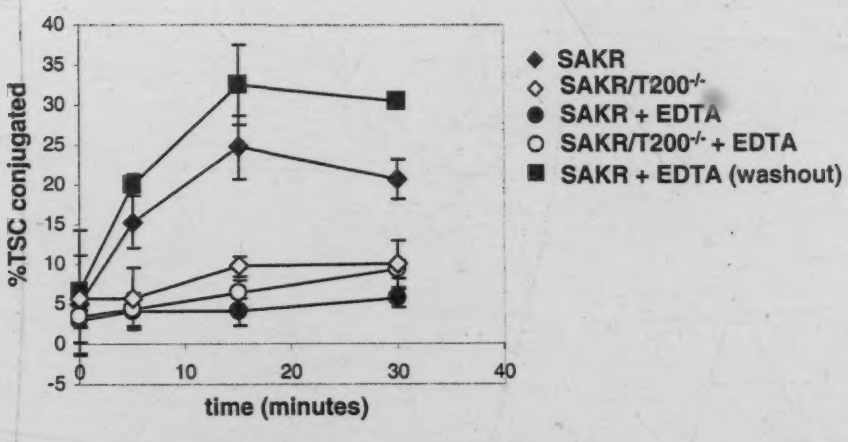


Figure 7: Adhesion between SAKR and TSC is cation dependent. SAKR (or SAKR/T200$^{-/-}$) cells and TSC were incubated in 5mM EDTA for 30 minutes prior to cell mixing in the adhesion assay. The adhesion assay was performed in the presence of 5mM EDTA, and then adhesion was assessed by flow cytometry. As a control, EDTA was removed immediately prior to cell mixing (SAKR + EDTA (washout)). This figure represents an average of three separate experiments.

30 minute EDTA incubation to remove EDTA prior to the mixing step. SAKR cells treated in this manner were able to adhere to TSC to a similar level, if not to a higher level, as untreated SAKR cells. This indicates that the cells are not damaged by EDTA treatment and that the inhibition of adhesion between TSC and SAKR afforded by EDTA is completely reversible.

Adhesion between SAKR and TSC is inhibited in the presence of specific carbohydrates in a dose-dependent manner.

Since the adhesion we observed between SAKR and TSC exhibited dependence on divalent cations we proceeded to test whether this adhesion could be inhibited through the addition of exogenous carbohydrates, as the ligands for lectins are the carbohydrate moieties present on glycoproteins. A number of different carbohydrates were tested, including glucose, mannose, lactose, laminarin, glucose-6-phosphate and mannose-6-phosphate at a concentration of 100mM, except for laminarin, which was used at a concentration of 100µg/mL. We found that adhesion was inhibited in the presence of glucose-6-phosphate and mannose-6-phosphate, and levels of adhesion in the presence of all other carbohydrates tested were indistinguishable from adhesion in the absence of carbohydrates (figure 8a). Further, we were able to show that the inhibition of adhesion in the presence of glucose-6-phosphate and mannose-6-phosphate was dose-dependent, as decreasing concentrations (100mM, 50mM and 25mM) inhibited adhesion to lesser degrees (figure 8b).

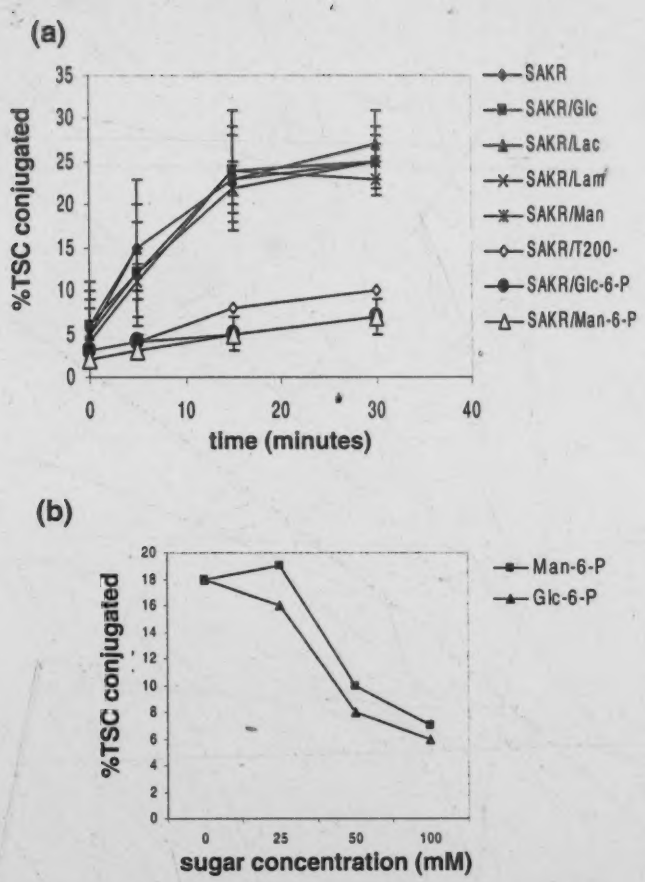


Figure 8: Adhesion between SAKR and TSC is inhibited by carbohydrates. (a) SAKR and TSC were incubated in the presence of 100mM carbohydrate (laminarin: 100µg/mL) prior to mixing in adhesion assay. Adhesion assays were performed in the presence of the carbohydrate, and adhesion was assessed by flow cytometry. This figure represents an average of three separate experiments. (b) Adhesion assays were performed between SAKR and TSC in the presence of varying concentrations of glucose-6-phosphate or mannose-6-phosphate (0mM-100mM).

Adhesion is not observed between BW5147 cells and TSC in the presence or absence of CD45 expression.

As we were able to show CD45-dependent adhesion using the SAKR and SAKR/T200$^{-/-}$ variants, we decided to test whether or not the same could be seen using another cell line. BW5147 is another T-lymphoma cell line for which we also have CD45$^{+/+}$ and CD45$^{-/-}$ variants. Surface staining using the pan-specific anti-CD45 mAb I3/2 confirms that CD45 is only expressed on the CD45$^{+/+}$ variant. (figure 9a). No adhesion above the level of the SAKR/T200$^{-/-}$ cells and TSC was observed between BW5147 cells and TSC using our adhesion assay, regardless of the CD45 expression status of the BW5147 cells (figure 9b). This does not confirm the involvement of CD45 in the adhesion that we observed between SAKR and TSC, as in that system CD45$^{+/+}$ cells were able to adhere to TSC while CD45$^{-/-}$ variants were not.

SAKR and BW5147 cells exhibit differences in PNA binding.

One possible explanation for the lack of adhesion of BW5147 cells is that they have a mutation in the pathway leading to mature core-2 O-linked glycosylation. Specifically, the cells are deficient in the enzyme C2GnT (core 2 β-1,6-*N*-acetylglucosaminyltransferase), which is required for the addition of the core 2 branch on O-linked glycans, and thus for the development of mature O-linked glycans (40). As we speculate in our hypothesis that specific carbohydrate-lectin interactions are responsible for the CD45-dependent

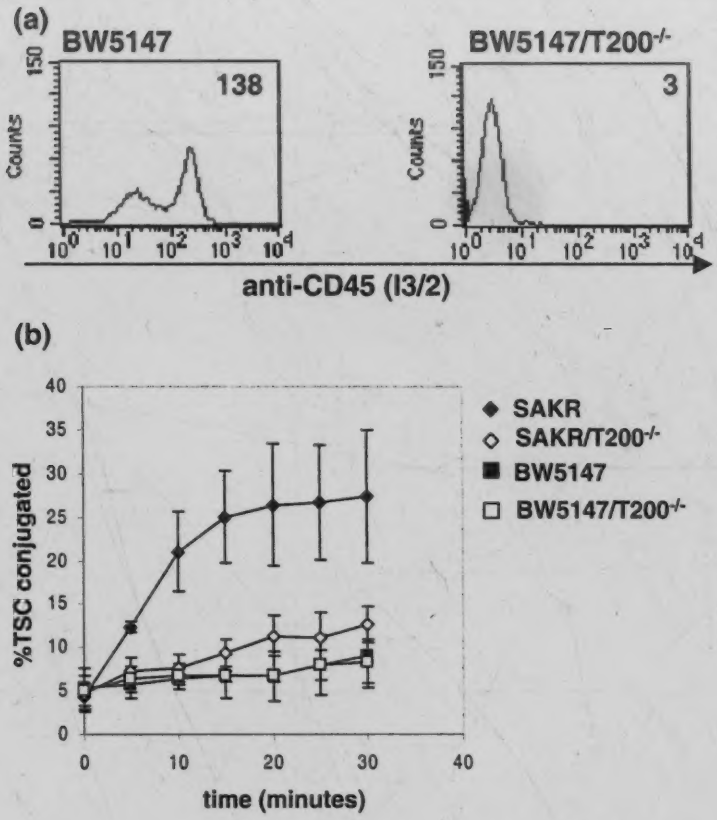


Figure 9: BW5147 cells do not adhere to TSC regardless of CD45 expression. (a) Surface stain of BW5147 and BW5147/T200$^{-/-}$ cells using the pan-specific anti-CD45 mAb I3/2. Mean fluorescence intensity (MFI) of CD45 staining for each cell type is indicated. (b) Adhesion assays were performed between BW5147 or BW5147/T200$^{-/-}$ cells and TSC as described, and compared with results from similar adhesion assays between SAKR and TSC.

adhesion we went on to test the possibility that differences in this O-linked glycosylation between the SAKR and BW5147 cells could account for the observed differences in adherence to TSC. A panel of FITC-labelled plant-derived lectins were utilized in a flow cytometric surface staining screen to compare the lectin binding profiles of SAKR and BW5147 cells. Peanut Agglutinin (PNA) is a plant-derived lectin that recognizes immature glycans, and more specifically glycans that have not had the terminal sialic acid added, these unsialidated glycans can be considered immature, as the addition of sialic acid is the final event in the normal development of glycan modifications. This screening process revealed that there was a difference between SAKR and BW5147 cells in their ability to bind PNA. BW5147 exhibited a high degree of PNA staining compared with SAKR cells (fig 10a). This result is consistent with the inability of BW5147 cells to generate mature O-linked glycans, and presented a potential explanation for the differences in adhesion to TSC that was observed between the SAKR and BW5147 cells.

Adhesion between SAKR and TSC is sialic acid independent.

In order to explore the possibility that sialic acid moieties are important in CD45-dependent adhesion between SAKR and TSC we treated SAKR cells with neuraminidase (also known as sialidase), which removes terminal sialic acid moieties and effectively increases the ability of treated cells to bind PNA. If sialic acid is important in the observed adhesion between SAKR and TSC, neuraminidase treatment would be expected to abolish this adhesion. SAKR

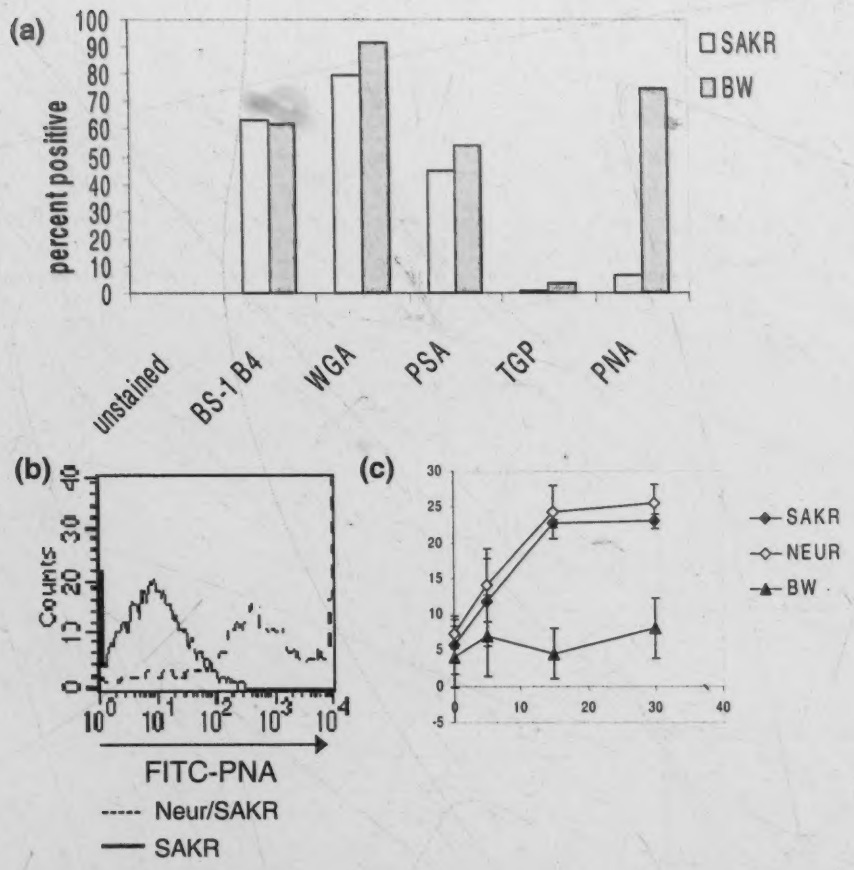


Figure 10: Adhesion between SAKR and TSC is not sialic acid dependent. (a) Surface stain of SAKR and BW cells using a panel of FITC-labeled plant lectins, all with different carbohydrate specificities (BS1-B4 recognizes terminal α-D-galactosyl residues; WGA recognizes N-acetyl-β-D-glucosyl residues; PSA recognizes terminal α-D-mannosyl residues; TGP recognizes α-L-fucose; and PNA recognizes immature unsialidated glycans). (b) Surface PNA-FITC stain of SAKR before (solid line) and after (broken line) neuraminidase treatment. (c) adhesion assay to compare adhesion between SAKR and TSC before and after SAKR were treated with neuraminidase (NEUR = neuraminidase-treated SAKR cells).

cells were treated with neuramindase and then mixed with TSC to test their ability to form conjugates in the adhesion assay. The level of adhesion between neuraminidase-treated SAKR and TSC was indistinguishable from the level of adhesion observed between untreated SAKR and TSC (figure 10c). Surface staining of SAKR cells with FITC-PNA was done to ensure that the neuraminidase treatment was successful. As expected, the neuraminidase treatment resulted in increased binding of FITC-PNA to the SAKR cells (figure 10b). As there was no change in the ability of SAKR to adhere to TSC following neuramindase treatment we concluded that this adhesion is not mediated by sialic acid moieties on the external domain of CD45. However it is still possible that other differences in carbohydrates could be responsible for the differences in adhesion observed between SAKR and BW5147 cells.

Adhesion is not observed between SAKR or BW5147 and the macrophage cell line P388D1.

The thymus is composed of many types of cells, including epithelial cells, dendritic cells, fibroblasts and macrophages, all of which could potentially be involved in adhesion with developing thymocytes. In order to determine whether or not the adhesion patterns we had observed with the cultured TSC was specific for those cells we made use of the macrophage cell line, P388D1. Our adhesion assay was repeated using the macrophage cell line P388D1 in place of the TSC. In this assay SAKR and BW5147 along with their CD45-deficient variants (SAKR/T200$^{-/-}$ and BW5147/T200$^{-/-}$) were used. No adhesion was observed with

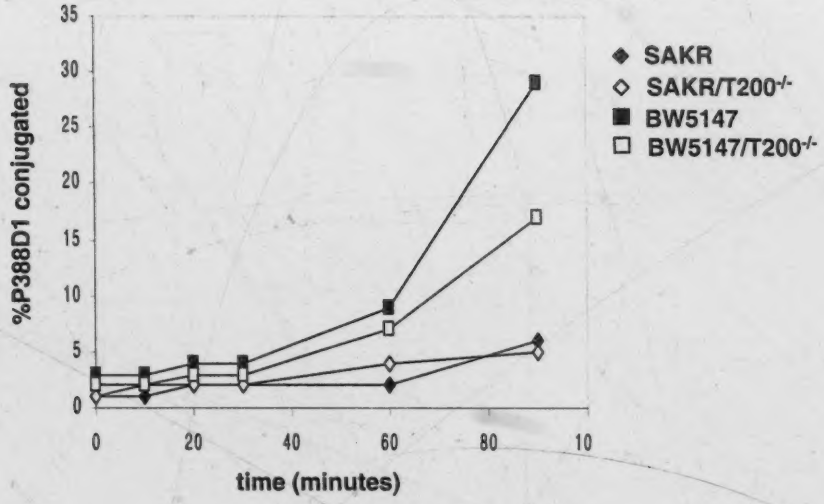


Figure 11: SAKR or BW5147 cells do not adhere to the macrophage cell line P388D1. Adhesion assay was performed between SAKR or BW5147 cells and P388D1 cells (a macrophage cell line). Formation of double positives at the later time points can be attributed to uptake of membrane fragments from the SAKR or BW5147 cells by the P388D1 cells.

any of the T-lymphoma cell lines (figure 11). At very late time points (90 minutes), some increase in the percent P388D1 conjugated was observed, but this is not the result of a direct increase in conjugate formation, as there is no development of a discrete double positive population in the FL-1 vs. FL-2 FACS plot (data not shown). More likely this increase in percent P388D1 can be attributed to the active uptake of membrane fragments of the T-lymphoma cells by the macrophage cell line.

***Ex vivo* thymocytes from wild-type and CD45$^{-/-}$ mice adhere to TSC to similar levels.**

We were very interested in this adhesion between the SAKR and TSC, in particular in the possibility that this interaction may represent a physiologically relevant interaction that occurs between developing thymocytes and thymic stromal cells during the process of thymic selection. To explore this possibility we obtained thymi from both CD45$^{+/+}$ and CD45$^{-/-}$ C57BL/6 mice. Thymocytes were isolated from these thymi, stained, and used in the adhesion assay in the same manner as the T-lymphoma cell lines were used. Surface stains of the isolated thymocytes with the pan-specific anti-CD45 mAb I3/2 showed that the thymocytes displayed the expected CD45 expression patterns (figure 12a). Also, double staining with anti-CD4 and anti-CD8 mAbs were consistent with expected thymic developmental patterns for these mouse lines (figure 12b). When these cells were subjected to adhesion assays with TSC, however, both the CD45$^{+/+}$

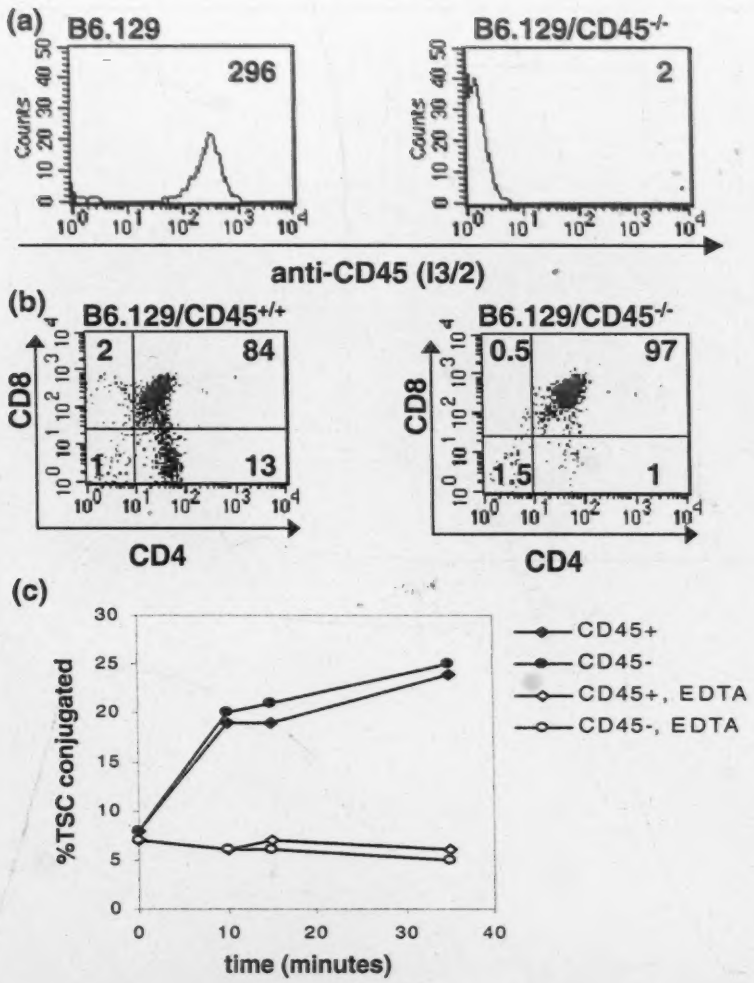


Figure 12: *Ex vivo* thymocytes do not exhibit differences in binding with CD45 expression. Thymocytes were extracted from B6.129 ($CD45^+$) and B6.129/$CD45^-$ mice and surface stained (a) for CD45 using the mAb I3/2 (MFI is indicated) or (b) for CD4 and CD8 using mAbs conjugated to FITC and PE, respectively. Percentage of cells appearing in each quadrant is indicated. (c) Results of adhesion assay between TSC and $CD45^{+/+}$ or $CD45^{-/-}$ thymocytes in the presence or absence of 5mM EDTA.

and CD45$^{-/-}$ thymocytes displayed similar adhesion to the TSC, as in both cases approximately 20% of TSC were involved in conjugates (figure 12c).

SAKR/TSC adhesion is not inhibited in the presence of blocking mAbs against LFA-1.

We sought to further characterize the SAKR/TSC interaction by looking at the possibility that the interaction could be mediated by integrins expressed on the surface of the T-lymphoma cells. The most abundant integrin expressed on T cells is leukocyte functional antigen (LFA-1). LFA-1 is believed to play a role in adhesion during the initiation of T cell signaling through an interaction with ICAM-1. LFA-1 is expressed to similar levels on SAKR and SAKR/T200$^{-/-}$ cells (figure 13a). We made use of a mAb against LFA-1 which had previously been shown to block the LFA-1/ICAM-1 interaction (11). Incubation of SAKR and TSC with 10ug/ml of anti-LFA-1 (M17/5.2) prior to mixing in the adhesion assay, and maintaining this concentration of mAb throughout the assay did not inhibit adhesion between SAKR and TSC (figure 13b).

SAKR/TSC adhesion is inhibited in the presence of blocking mAbs against VLA-4.

There are numerous integrins that could potentially mediate adhesion between SAKR and TSC, so we decided to try blocking with an antibody against VLA-4 (CD49d, $\alpha_4\beta_1$-integrin), since it is expressed by thymocytes (86). We

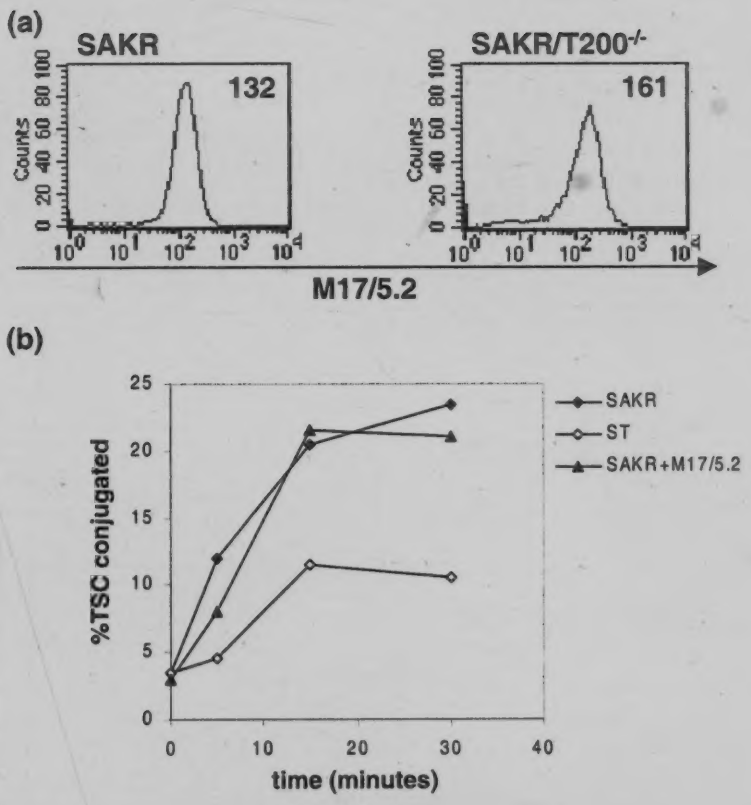


Figure 13: Adhesion between SAKR and TSC is not inhibited in the presence of blocking anti-LFA-1 antibodies.
(a) surface stain of SAKR and SAKR/T200$^{-/-}$ using the anti-LFA-1 mAb M17/5.2. MFI is indicated. (b) Adhesion assays were performed between SAKR and TSC in which both cell types were incubated for 30 minutes with 10µg of M17/5.2 in PBS/4% DCS prior to cell mixing. Antibody concentration was maintained throughout the assay. This experiment is representative of two separate experiments.

found that adhesion between SAKR and TSC was inhibited in the presence of 10μg/mL of the anti-VLA-4 mAb PS/2 but not in the presence of 10μg/mL of the anti-LFA-1 mAb M17/5.2 or the pan-specific anti-CD45 antibody I3/2 (figure 14b). Separate experiments using various anti-CD45 antibodies as potential blocking agents (M1/9.3.4, M1/8.9, MB23G2, and MB4B4) either individually or as a cocktail, showed that these anti-CD45 antibodies were also unable to interfere with SAKR/TSC adhesion (data not shown). Surface staining analysis of SAKR and SAKR/T200$^{-/-}$ cells with PS/2 was used to determine relative VLA-4 expression levels on both of these cell types revealed that VLA-4 was only expressed on the SAKR cells, and not on the CD45$^{-/-}$ SAKR/T200$^{-/-}$ cells (figure 14a).

SAKR/T200$^{-/-}$ cells exhibit different adhesion properties depending on time spent in culture.

Throughout the course of this study there was some inconsistency in the levels of adhesion achieved by the SAKR/T200$^{-/-}$ cells with TSC that we could not explain. A potential explanation presented itself, however, with the observation that the adhesion observed between SAKR/T200$^{-/-}$ cells and TSC is very different depending upon how long the SAKR/T200$^{-/-}$ cells are cultured prior to the assay. This difference is independent of cell density, as cultures were seeded at concentrations that would allow cell density to be similar between cells cultured for different lengths of time. Cell density was approximately 2-6x10^6 cells/mL at the time of assay. SAKR/T200$^{-/-}$ cells cultured for one day do not adhere to TSC,

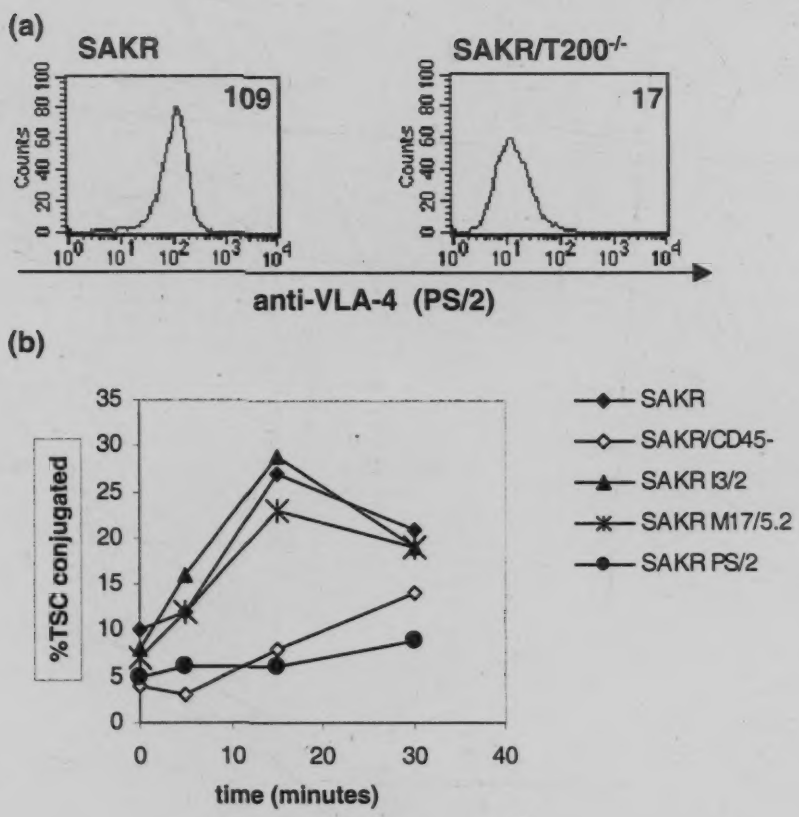


Figure 14: Adhesion between SAKR and TSC is inhibited in the presence of blocking anti-VLA-4 antibodies. (a) Surface stain of SAKR and SAKR/T200$^{-/-}$ for using the anti-VLA-4 mAb, PS/2. (b) Adhesion assays were performed between SAKR and TSC in which both cell types were incubated for 30 minutes with 10μg of PS/2 (anti-VLA-4), I3/2 (anti-CD45), or M17/5.2 (anti-LFA-1) in PBS/4% DCS prior to cell mixing. Antibody concentration was maintained throughout the assay. This experiment is representative of two separate experiments.

but the same cells cultured for three days adhere to TSC to the same levels as $CD45^{+/+}$ SAKR cells (figure 15a). We speculated, in light of the results of the PS/2 blocking studies, that perhaps expression of VLA-4 was upregulated on the surface of the $SAKR/T200^{-/}$ after a certain amount of time in culture. However, PS/2 surface stain of cells cultured for both one day and three days revealed nodifference in surface expression levels of VLA-4. Thus the molecular basis for the change in adhesion ability of $SAKR/T200^{-/-}$ cells is unknown at this time (figure 15b).

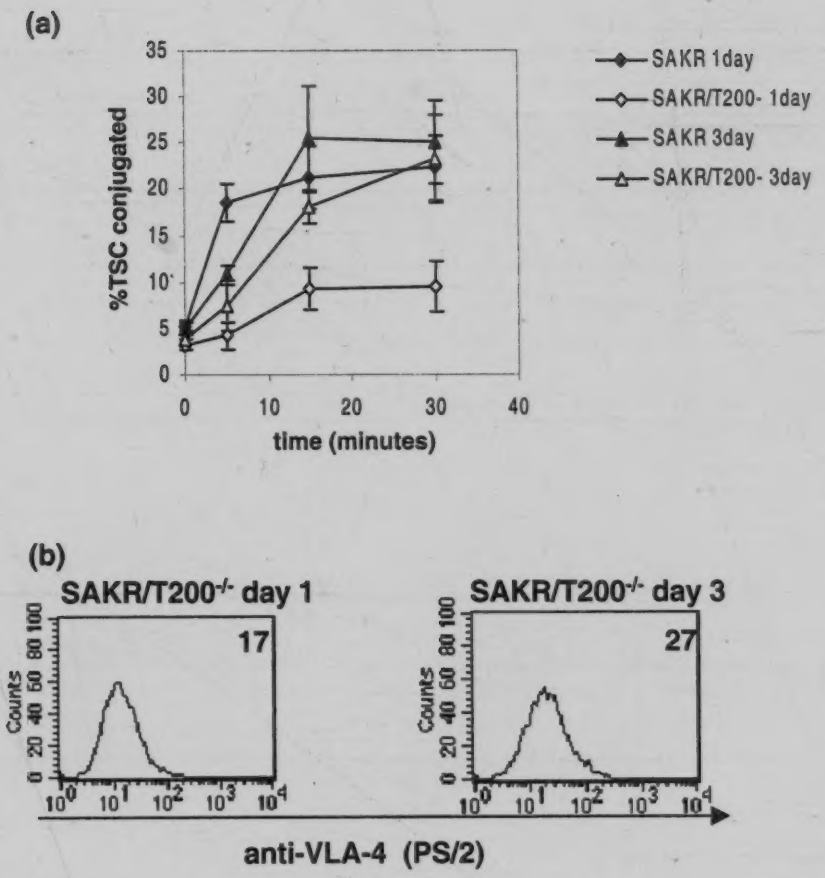


Figure 15: SAKR/T200$^{-/-}$ cells exhibit different adhesion properties depending on time spent in culture. (a) SAKR and SAKR/T200$^{-/-}$ cells were cultured either for 1 day or 3 days prior to adhesion assay. Cells were seeded with different cell numbers so that on the day of the assay they would be of approximately equal densities (~5X10^6 cells/mL). (b) surface stain for VLA-4 using the mAb PS/2.

CHAPTER IV

DISCUSSION

Our original hypothesis states that the extracellular domain of CD45 is important in mediating cell-cell interactions between thymic stromal cells and developing thymocytes during thymocyte development through carbohydrate – lectin interactions involving the glycosylation modifications on the external domain of CD45 and lectins on the surface of thymic stromal cells. Some of our data support this hypothesis, in that we do observe apparent CD45-dependent adhesion between SAKR and TSC, and this adhesion can be inhibited both by removal of calcium and by addition of exogenous carbohydrates. However, we also observe that adhesion can be inhibited by antibodies against the integrin VLA-4. Together these results are consistent with a thymocyte development model where numerous molecular interactions are involved in adhesion leading to signals that contribute to the selection of thymocytes.

The results presented herein have confirmed previous observations made in our lab, and have shown that, at least in SAKR cells, adhesion to TSC seems to be affected by lack of CD45 expression. Adhesion was seen between TSC and $CD45^{+/+}$ SAKR cells, but not $CD45^{-/-}$ SAKR cells, suggesting that adhesion in this system may indeed be CD45-dependent. There are numerous ways to interpret this result. It is possible that adhesion is mediated directly by interactions between the external domain of CD45 and an unknown counter-receptor on the surface of the TSC. Alternatively, CD45 may be involved in

regulation of adhesion in an indirect manner by regulating expression or activation state of other adhesion molecules. Another possibility is that the absence of CD45 simply disrupts the glycocalyx of the lymphocyte, and that this disruption changes the ability of the cell to interact normally with other cells. Still another potential interpretation is that the adhesion is not dependent upon CD45, but that there is another unknown difference between the $CD45^{+/+}$ and $CD45^{-/-}$ variants of SAKR. There are still many pieces missing to the CD45 puzzle. Hopefully in time more of these pieces will be defined allowing the big picture to finally emerge. The thing that is quite clear at this time is that CD45 is globally important to the function of T cells, as processes as divergent as integrin-mediated adhesion and thymocyte development are adversely affected by the absence of CD45.

The characteristics of the external domain of CD45, in that it is a very large, highly glycosylated and closely regulated domain make it very tempting to speculate that it is regulated by a specific ligand that can facilitate its role in lymphocyte function. In fact, based on homology to the phosphatase PTP1B, CD45 is known as a receptor-linked protein tyrosine phosphatase (20). If CD45 is indeed modulated by a receptor, it is unusual that there has been no success in the identification of this molecule, despite the many years that have been spent by many people in trying to find this elusive ligand. It is possible that CD45 does not have a single specific ligand. The available evidence seems to point in the direction of CD45 being involved in numerous intertwining pathways, each the product of extremely complex and, in many cases, poorly defined regulation.

The Adhesion Assay

Our approach to the study of CD45-dependent adhesion has been a flow cytometry-based adhesion assay. This assay, like most, has both strong points and limitations. It is particularly good for the detection and quantification of adhesion between two different cell types, as the flow cytometer is capable of counting hundreds of fluorescent events per second. Also, we are able to choose the characteristics of the cells that we use for adhesion, and have the option of adding a wide variety of potential blocking agents to the assay.

However, the assay requires that all cells are in suspension. Under physiological conditions, thymic stromal cells are not in suspension, but instead are involved in a complex 3D formation through which the developing thymocytes migrate and interact with the stromal elements. Removal of stromal cells from this environment could cause changes away from their normal physiological condition. Thus our assay system is useful as a preliminary tool to detect interactions that may be relevant physiologically, but any interactions that are detected in this system should be tested further using a more physiological system such as fetal thymic organ culture (FTOC).

Another shortcoming of our assay system is the cultured thymic stromal cells. These were isolated from thymus according to a protocol designed to specifically isolate thymic epithelial cells. However, we have been unable to detect antibody staining using commercial antibodies against known thymic epithelial cell markers. As such we cannot be absolutely sure that the cells are

epithelial in nature. It is possible that the cells are another type of thymic stromal cell, or even that the cells have differentiated in culture to become very different from the cells found in the thymus.

Our thymic stromal cells have at least some characteristics of epithelial cells, as they are able to grow in the presence of D-valine. Cells can only directly utilize the L-isomer of valine, so if only the D-isomer is provided, an isomerization reaction must occur. The ability of our cells to continue to grow in the presence of D-valine shows that these cells are not fibroblast cells, another cell type that could potentially grow out of the thymus. Fibroblasts are incapable if isomerization of D- to L-valine as they lack the enzyme D-amino acid oxidase (42). However, as we have been unable to detect surface staining for known epithelial cell markers, we will continue to refer to these cells as thymic stromal cells.

It is important to keep these limitations in mind, but it is also important to remember that this assay is intended to act as a screen for potential interactions involving CD45 that may occur in the thymus. Thus it is appropriate to use these thymic stromal cells to screen for interactions as long as any findings are subsequently confirmed, either in a more physiological system or in a system involving purified molecules.

Specific carbohydrates are capable of blocking SAKR/TSC adhesion

Our results show that the interaction between $CD45^{+/+}$ SAKR and TSC can be inhibited by EDTA, indicating that the interaction is cation dependent. We

were also able to inhibit the interaction through the addition of either glucose-6-phosphate or mannose-6-phosphate. These results are consistent with our hypothesis, as lectin interactions are often cation dependent, and can often be inhibited by specific carbohydrates. But what could this mean in terms of thymocyte development? One thing that is certain about the process of thymocyte development is that cell-to-cell interactions are extremely important, and that TCR/MHC recognition plays a central role. These interactions, however, are often of low affinity and require accessory molecules to tether the cells together so that the TCR/MHC interaction can take place. In dendritic cells, DC-SIGN provides this important accessory interaction. The interaction is antigen independent, and occurs in a cation- and carbohydrate-dependent manner. Perhaps the CD45 external domain is acting analogously in immature thymocytes to tether thymocytes to thymic stromal elements to allow the necessary developmental steps to take place. This could explain why the $CD45^{-/-}$ mice display a block in the DP→SP transition, as this is the stage where TCR/MHC interactions are particularly important in the processes of positive and negative selection.

Inhibition by the specific carbohydrates glucose-6-phosphate and mannose-6-phosphate is somewhat difficult to explain without a detailed understanding of the glycosylation status of the particular CD45 molecules involved in the SAKR/TSC interaction. Such a characterization has numerous intrinsic difficulties, and as such it is not feasible to undertake this line of experimentation at this time. These problems are similar to those encountered

with the confusion surrounding the determination of CD45 isoform expression. The sheer variability and abundance of the CD45 molecules expressed on the surface of even a single cell would make it very difficult to separate any relevant populations from the large cellular pool.

Sialic acid does not appear to be responsible for SAKR/TSC adhesion

We did, however, have a cell line in the lab with the potential to allow us to examine the involvement of at least some types of glycosylation. This cell line is the BW5147 (BW) cell line, of which we also have $CD45^{+/+}$ and $CD45^{-/-}$ variants. Originally we started working with these cells with the intention of confirming our SAKR/TSC adhesion results. This did not occur, as BW cells do not adhere to TSC, regardless of whether or not they express CD45. Although this finding was surprising and somewhat disappointing at first, it was encouraging to discover that BW cells have a very different glycosylation pattern than SAKR cells do, as BW do not express the enzyme core 2 β-1,6-*N*-acetylglucosaminyltransferase (C2GnT), an enzyme required for the normal development of branched O-linked glycosylation (76). O-linked glycans are important for specific recognition by lectins, for example, galectin-1, selectins and peanut agglutinin (PNA) have been shown to bind to carbohydrate moieties within these modifications (65). Thus it is possible that the adhesion observed between SAKR and TSC is mediated by carbohydrates within the O-linked glycan modifications, and that this is the reason for the differences we see between these cell lines.

A surface stain comparison of BW and SAKR cells using various FITC-labeled plant lectins revealed that there was indeed a difference in staining between the two cell lines for PNA, in that BW are positive and SAKR are negative for PNA staining. This PNA binding pattern is consistent with BW lacking the C2GnT enzyme, as PNA binds cells with immature glycosylation patterns.

The addition of sialic acid to O-linked glycans is normally the final event in the development of these modifications, so mature and immature glycans can broadly be defined by the presence or absence of sialic acid on their surface. PNA binds unsialylated glycans but not glycans containing sialic acid. Perhaps the binding between SAKR and TSC is mediated by sialic acid modifications on the extracellular domain of CD45. This sort of interaction has been seen before, as CD22 is a sialic acid binding lectin capable of binding CD45, as well as other cell surface molecules (102). Neuraminidase is an enzyme that removes sialic acid from the surface of cells, resulting in an increase in PNA binding compared to untreated cells. If the adhesion is indeed mediated by sialic acid we would expect that neuraminidase treatment would abolish SAKR/TSC interaction. This was not the case. Neuraminidase treatment had absolutely no effect on the level of adhesion observed between SAKR and TSC, even though PNA binding was significantly increased. This result suggests that sialic acid is not responsible for SAKR/TSC adhesion. However, as BW5147 cells are lacking the enzyme required to develop mature O-linked glycosylation, there are numerous potential carbohydrate differences between SAKR and BW5147 that could contribute to

the difference we observed in their abilities to form conjugates with TSC. The differences between SAKR and BW cells that leads to the differences in adhesion with TSC, then, are still unknown at this time.

Adhesion is observed between thymocytes and TSC regardless of CD45 expression

Adhesion assays between freshly isolated thymocytes and TSC yielded equally disappointing results, as no difference was observed in adhesion between TSC and either $CD45^{-/-}$ or $CD45^{+/+}$ thymocytes. However, unlike the BW results, adhesion was observed between thymocytes and TSC regardless of whether or not CD45 was expressed. This is a very complex system, and a negative result in this situation does not rule out the possibility of CD45 being involved in adhesion between immature thymocytes and TSC. First it is important to keep in mind that the populations of thymocytes being examined are very different, as the lack of CD45 leads to a block in thymocyte development resulting in a very different surface phenotype than that of the wildtype thymocytes. Thus it may be that the adhesion we see in each of these types of thymocytes is mediated by different molecules. To see practically identical levels of adhesion mediated by completely different pathways would seem fairly coincidental. Perhaps a more reasonable explanation for this result would be that it is possible that in our assay system there is another molecule, present on both types of thymocytes, that is able to mediate adhesion with TSC in a CD45-

independent manner. In this case, even if CD45 is able to mediate adhesion we may be unable to detect that adhesion.

Integrin involvement

Perhaps the most well-known example of integrin involvement in cell-to-cell contact is in the formation of the immunological synapse at the contact point between a T cell and an antigen presenting cell (APC) (48). Ultimately this contact requires a prolonged interaction between the TCR and its specific MHC/peptide complex. The APC may display numerous MHC/peptide complexes, and as such it is very possible that only a very few of these complexes could be recognized by the specific TCR carried by the T cell. The chances of antigen-specific interactions are greatly increased by an initial interaction that occurs between the integrin LFA-1 (leukocyte functional antigen-1) and ICAM-1 (intercellular adhesion molecule-1). These interactions hold the T cell and APC in close enough contact for the TCR to sample the APC surface for MHC/peptide recognition. If such recognition does occur there is a reorganization at the site of contact leading to central clustering of the TCR/MHC/peptide complexes. The LFA-1/ICAM-1 molecules form a ring around the central antigen-specific cluster, and this contact can be maintained for extended periods of time, sufficient for the transmission of TCR-mediated signals leading to T cell activation (75).

In light of the important role played by the LFA-1/ICAM-1 interaction in T cell activation, it was important for us to rule out the possibility that the adhesion

we observed between SAKR and TSC was due to interactions involving LFA-1. We thought this would be unlikely, as there is no difference in levels of surface expression of LFA-1 on the surfaces of the $CD45^{+/+}$ and $CD45^{-/-}$ variants of SAKR. We utilized the mAb M17/5.2, an antibody previously shown to block CTL interactions in our laboratory (11). The results showed that anti-LFA-1 does not block adhesion between SAKR and TSC in our adhesion assay.

The suggestion that CD45 may be important in the regulation of integrin-mediated adhesion is not without precedent and, in fact, studies of macrophages from CD45 $exon6^{-/-}$ mice have revealed a dysregulation of Src-family kinases associated with integrin mediated adhesion in these cells (84). As well, CD45-deficient Jurkat cells (human T cell line) displayed enhanced adhesion to fibronectin through VLA-5 ($\alpha_5\beta_1$) (96). The results of both of these studies suggest that CD45 is involved in negative regulation of integrin-mediated adhesion, as the adhesion is increased in the absence of CD45. This is opposite to what we see, as in our system adhesion only occurs in the presence of CD45, and can be blocked by anti-VLA-4 antibodies.

There are some important differences between our study and the ones mentioned above. The most notable of these differences is the target of adhesion being studied. The macrophage and Jurkat studies are looking at adhesion to a surface, such as a tissue culture plate or immobilized fibronectin, while our study is concerned with cell-to-cell adhesion. This is important because adhesion to a surface generally occurs for the purposes of cell motility or anchoring, and involves the formation of focal adhesions, which are regions of

concentrated integrins, tyrosine phosphorylated proteins and cytoskeletal proteins that form within a cell at points of contact between the cell membrane and the extracellular matrix. While many of the same molecular elements may be present at sites of cell-to-cell interaction, the regulation of these elements may be very different. The purpose of interactions between cells is most often the transmission of signal from one cell to the other, and as such the requirements for integrin-mediated adhesion can be very different. While the function of integrins in focal adhesions is to provide a link between extracellular signals and the cytoskeleton to allow the cell to move, integrins in cell-cell contact areas are more likely to be involved in tethering the cells together so that pertinent signals can be exchanged.

The enhanced adhesion observed in CD45-deficient Jurkat cells was mediated by VLA-5, while the adhesion we report is blockable by anti-VLA-4. We also observe that VLA-4 is only expressed on our $CD45^{+/+}$ SAKR cells. Together these results suggest that CD45 may influence adhesion through different integrins under different conditions. VLA-4 and VLA-5 are similar in that both can bind to fibronectin, a common component of the extracellular matrix. However, VLA-4 is capable of binding to VCAM-1 as well. Previous studies have shown that early DP thymocytes express VLA-4, but that VLA-5 is not expressed until a later stage of development (87). Further studies indicated that VCAM-1 is expressed in the cortex of the thymus, while FN is found mainly in the medulla. Also, these same studies showed that adhesion between thymic epithelial cells and thymocytes was mediated by the VLA-4/VCAM-1 interaction (86). These

results, taken together are consistent with the involvement of CD45 in the regulation of VLA-4-mediated adhesion in immature DP thymocytes. This presents an intriguing potential explanation for the thymic developmental phenotype of $CD45^{-/-}$ mice, as the major developmental block in these mice is at the DP→SP transition. Perhaps CD45 is required for the regulation of an essential VLA-4-mediated interaction during the TCR-mediated events that occur during positive and negative selection.

Conclusions

The results of the present study do not lead us to any simple answers, only to more questions. We set out to examine potential CD45-dependent carbohydrate-based interactions between lymphoma cells and unknown lectins on thymic stromal cells, with the hope that our findings may relate to the lymphostromal interactions that occur during thymocyte development. Our results show that the CD45-dependent adhesion we observe between $CD45^{+/+}$ SAKR cells and TSC displays characteristics of a lectin interaction in that the adhesion requires the presence of divalent cations and can be inhibited in the presence of specific carbohydrates in a dose-dependent manner. These results are consistent with a direct, lectin-based interaction involving the external domain of CD45. However, this result also does not rule out an indirect interaction involving adhesion molecules regulated by CD45. Further results from our study show that antibodies specific for the α4β1 integrin VLA-4 block adhesion between $CD45^{+/+}$ SAKR cells and TSC. This result seems to support the idea

that the CD45-dependent adhesion observed is in fact an indirect result of CD45 integrin regulation, but also leaves open the possibility that the observed differences in adhesion are independent of CD45 expression. It may be that CD45 functions a central regulator in numerous pathways involved in cell-to-cell adhesion during the development of thymocytes.

Future directions

It will be important in the future to determine whether this adhesion is being mediated by direct binding with the extracellular domain of CD45, or if the observed adhesion is a result of regulation by CD45. The present system is not appropriate for these types of studies, as it is not possible to determine the specific molecules involved in cell-to-cell adhesion. Perhaps a more relevant system would be to label and purify CD45 from cells and then use this labeled CD45 in surface binding studies with thymic stromal cells to see if CD45 is capable of direct binding to these cells. However, it may be difficult to obtain sufficient amounts of protein. Expression of CD45, or even of the extracellular domain of CD45 in another cell type could be useful in that it would be easier to obtain large amounts of protein. The problem with this approach is that the glycosylation pattern of CD45 may be centrally important for binding, and glycosylation generally differs from cell type to cell type. Thus, although binding studies using artificially expressed or purified CD45 molecules could provide useful information regarding the interactions we have seen, these studies may be very difficult to execute.

Binding through the integrin VLA-4 should be examined further. It is not clear from our present study whether or not this interaction is mediated by VLA-4/VCAM-1 or VLA-4/FN interaction. A very simple way to start to determine the basis of this interaction would be to look for VCAM-1 and FN on the surface of the TSC, and to use antibodies against these molecules as potential blocking agents to adhesion. Along with these studies, small peptide inhibitors could be used to block adhesion as well. The VLA-4 binding motifs for FN and VCAM-1 are known, and they are different from one another. Using peptides containing these binding motifs as adhesion blockers could also be useful in understanding the VLA-4 involvement in adhesion.

We do not see adhesion between SAKR/T200$^{-/-}$ cells and TSC. This may be due to lack of CD45 expression, but it may also be due to lack of VLA-4 expression as we also do not detect surface staining for VLA-4 in these cells. It may be useful to transfect these cells with VLA-4 and then examine their ability to adhere to TSC.

The differing abilities to adhere that we observe between SAKR and BW could provide useful clues as to the nature of the SAKR/TSC adhesion. Examination of the glycosylation patterns of the two cell types, either through identification of the carbohydrates that are present or through inhibition of various carbohydrate pathways could tell us whether or not it is indeed differences in glycosylation that are responsible for the differences in adhesion. It would also be a good idea to look at differences in VLA-4 on the surface of these 2 cell types. Whether or not VLA-4 is expressed on BW should be determined, and if it

is expressed, the activation state of this molecule should be determined. If a particular form of VLA-4 is required for adhesion, it may be possible to induce VLA-4-mediated adhesion in BW cells by providing activators for this molecule.

Finally, the results of this study should be applied to studies in FTOC. Ultimately, if the adhesion that we observe is important to thymocyte development it should be possible to block thymocyte development in normal thymocytes using the blocking agents that block adhesion in this system. The studies reported here should act as a starting point for further studies, both in more physiological and more biochemical systems.

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